PRODUCTION OF CELLULOLYTIC ENZYMES UNDER NONASEPTIC AND NONSTERILE CONDITIONS

FINAL REPORT

prepared for

NATIONAL RESEARCH COUNCIL CANADA

OTTAWA, ONTARIO K1A OR6

under

DSS CONTRACT No. 05SR. 31926-4-5027

SERIAL No. OSR84-00095

Principal investigator:

Dr. Anh LeDuy, Professor
Department of Chemical Engineering
Laval University
Sainte-Foy, Québec G1K 7P4

Project scientists:

Ms. Nicole Cauchon, M. Sc.

Ms. Louise Cournoyer, B. Sc.

Research assistants (part-time):

Ms Suzanne Boisvert

M. Jean-François Bourassa, B.Sc.

October 25, 1985

Unrestricted circulation

SUMMARY

An acidophilic cellulolytic fungus strain NC-11 (deposited as Patent Culture ATCC-20677 and recently identified as Penicillium ochrochloron) was used for the fermentative production of the extracellular cellulolytic enzymes, under nonsterile and nonaseptic conditions. The culture medium consisted of carbohydrate substrate in simple basal salt solution of K_2HPO_4 0,1%; $(NH_4)_2SO_4$ 0,1%; $NaNO_3$ 0,1% and $MgSO_4.7H_2O$ 0,016% at pH 2,0 (adjusted with H_2SO_4). The maximum cellulolytic activities of the culture filtrates obtained from batch fermentation in 10 ℓ (working volume) bioreactor were: 1 IU of xylanase, 0,15 IU of CMCase and 0,02 IU of FPase in CMC-xylan medium; 0,05 IU of xylanase, 0,015 IU of CMCase and 0,02 IU of FPase in newspaper medium. In CMC-xylan medium, both xylanase and CMCase production were growth-associated. In newspaper medium, only CMCase production was associated with growth. Under nonsterile and nonaseptic fermentation conditions, an unidentified yeast contaminant was developed in the bioreactor. However, yeast contamination did not affect the cellulolytic enzyme production in the presence of high concentrations of acidophilic fungus mycelium in the bioreactor. Continuous fermentation in the bioreactor using free cells of acidophilic fungus was not possible: the yeast contaminant overgrew due to its faster growth rate and the acidophilic fungus mycelium was washed out because of its slower growth rate. Cellobiose, Na-CMC, xylan, newspaper and sawdust from softwood had no inducing power over the synthesis and excretion of cellulolytic enzymes of acidophilic fungus. Several mutation tests on acidophilic fungus with UV radiation, X-ray radiation, ethyl methanesulfonate, N-methyl-N'-nitro-N-nitrosoguanidine and nitrous acid have not yet resulted in a mutant of acidophilic fungus with higher FPase activity.

TABLE OF CONTENTS

		Page
SUMMARY		i
1	INTRODUCTION	1
1.1	Definitions	1
1.2	Aseptic and sterile conditions for enzyme production and for enzymatic hydrolysis	1
1.3	Search for nonaseptic and nonsterile process	3
1.4	Acidophilic fungus and acidic process for the fermentative production of cellulolytic enzymes	4
1.5	R&D strategy and general objective	8
2	MATERIALS AND METHODS	11
2.1	Materials	11
2.1.1	Microoorganism	11
2.1.2	Chemicals	11
2.1.3	Bioreactor system	11
2.2	Experimental methods	16
2.2.1	Strategic plan	16
2.2.2	Enzyme production from various substrates	24
2.2.3	Induction	25
2.2.4	Mutation	27
2.2.4.1	UV radiation	27
2.2.4.2	X-ray radiation	28
2.2.4.3	Mutagens	28
2.2.4.4	Screening test for survival colonies	29
2.2.5	Batch fermentation	30
2.2.5.1	Inoculum preparation	30
2.2.5.2	Fermentation experiment	33
2.2.6	Continuous fermentation	33

2.3 Analytical methods 34

2.3.1 pH 34
2.3.2 Biomass concentration 34
2.3.3 Dissolved oxygen concentration, k_La and Q_{O_2} . . . 34
2.3.4 ATP concentration 35
2.3.5 Protein concentration 35
2.3.6 Enzyme activities 36
2.3.7 Contamination 40

3 RESULTS AND DISCUSSION 41

3.1 On the analytical methods 41

3.1.1 Protein concentration 41
3.1.2 DO concentration, k_La and Q_{O_2} 43
3.1.3 Enzyme activities 46

3.1.3.1 Precision of the FPase assay 46
3.1.3.2 Effect of dilution of the FPase assay . . 46
3.1.3.3 Negative values of enzyme activity . . . 49
3.1.3.4 Miscellaneous tests on enzyme assays . . 49

3.1.4 Screening tests for mutant selection 52

3.2 On the improvement of enzyme production by induction and by mutation 56

3.2.1 Enzyme production on various substrates 56
3.2.2 Induction 58
3.2.3 Mutation 58

3.3 On the kinetics of fermentation in bioreactors 74

3.3.1 Batch fermentation 74
3.3.2 Continuous fermentation 95

3.4 On the contamination problem 98

3.4.1 Contamination and contaminants 98
3.4.2 Identity of the acidophilic fungus 106
3.4.3 Suggested solutions for the contamination problem 109

4 CONCLUSIONS AND RECOMMENDATIONS. 111

4.1 Conclusions 111

4.2 Recommendations for future work 113

REFERENCES 115

APPENDICES

A Analytical procedures 120

A.1 ATP concentration 120
A.2 Protein concentration 124
A.3 Enzyme activities 125

B Tabulated results 133

C Identification of acidophilic fungus: ATCC Report 153

LIST OF TABLES

1.1 Axenic cultures potentially useful for the production of extracellular cellulolytic enzymes under nonaseptic and nonsterile conditions. 5

2.1 Chemicals used 13

2.2 Cellulolytic enzyme assay methods. 37

3.1 Precision of the FPase assay method. 47

3.2 Mutation tests with UV: Number of survival colonies 64

3.3 Mutation tests with X-ray: Number of survival colonies. . . 65

3.4 Mutation tests with EMS: Number of survival colonies. . . . 66

3.5 Mutation tests with NTS: Number of survival colonies. . . . 67

3.6 Mutation tests with nitrous acid: Number of survival colonies 68

3.7 Batch fermentation in bioreactors: Kinetic parameters . . . 85

3.8 Batch fermentation in bioreactors: Variation of CMCase activity-ATP concentration ratio 89

3.9 Batch fermentation in bioreactors: Maximal cellulolytic activities of culture filtrate 94

A1.1 Standard calibration curve for ATP 128

A2.1 Standard calibration curve for protein 130

A3.1 Standard calibration curves for glucose. 132

B.1 Effect of TCA precipitation time on the standard calibration curve for protein. 134

B.2 Effect of enzyme dilution on the measured enzyme activities. 135

B.3 Enzyme production from various substrates: Xylanase 136

B.4 Enzyme production from various substrates: CMCase 137

B.5 Enzyme production from various substrates: FPase. 138

B.6 Induction test with cellobiose 139

B.7 Induction test with Na-CMC 140

B.8 Induction test with xylan. 141

B.9 Induction test with newspaper. 142

B.10 Induction test with sawdust. 143

B.11 Batch fermentation in bioreactors: Variations of pH, temperature and DO concentration. 144

B.12 Batch fermentation in bioreactors: Variation of ATP concentration. 145

B.13 Batch fermentation in bioreactor: Variation of biomass concentration and ATP pool in CMC-xylan medium 146

B.14 Batch fermentation in bioreactors: Variation of xylanase activity 147

B.15 Batch fermentation in bioreactors: Variation of CMCase activity 148

B.16 Batch fermentation in bioreactors: Variation of FPase activity 149

B.17 Batch fermentation in bioreactors: Variation of protein concentration. 150

B.18 Batch fermentation in bioreactors: Variations of k_La and $Q_{O_2}C$ in newspaper medium 151

B.19 Batch fermentation in bioreactors: Variations of k_La and $Q_{O_2}C$ in CMC-xylan medium 152

LIST OF FIGURES

1.1 Strategy for process R&D 9

2.1 Pure culture of the acidophilic fungus NC-11 (ATCC-20677) grown in glucose and xylose at pH 2,0. 12

2.2 Detail of the bioreactor 17

2.3 Detail of the bioreactor 18

2.4 Bioreactor system. 19

2.5 Experiment strategic plan. 20

2.6 Experiments on the production of enzymes on various substrates. 26

2.7 Batch fermentation in bioreactors: Inoculum preparation . . 31

3.1 Effect of TCA precipitation time on the standard calibration curve for protein. 42

3.2 Typical dynamic gassing-out curve for DO concentration in CMC-xylan medium: Bioreactor 2A at 211 h. 44

3.3 Typical dynamic gassing-out curve for DO concentration in newspaper medium: Bioreactor 1B at 214 h. 45

3.4 Effect of enzyme dilution on the measured enzyme activities. 48

3.5 Miscellaneous tests on enzyme assays 50

3.6 Methods for screening of positive mutants. 54

3.7 Enzyme production from various substrates. 57

3.8 Induction test with cellobiose 59

3.9 Induction test with Na-CMC 60

3.10 Induction test with xylan 61

3.11 Induction test with newspaper 62

3.12 Induction test with sawdust. 63

3.13 Mutation tests with UV: Relation between FPase activity and released color of survival colonies. 69

3.14 Mutation tests with X-ray: Relation between FPase activity and released color of survival colonies. 70

3.15 Mutation tests with EMS: Relation between FPase activity and released color of survival colonies. 71

3.16 Mutation tests with NTG: Relation between FPase activity and released color of survival colonies. 72

3.17 Mutation tests with nitrous acid: Relation between FPase activity and released color of survival colonies 73

3.18 Batch fermentation in bioreactors: Variation of pH, temperature and DO concentration in newspaper medium 75

3.19 Batch fermentation in bioreactors: Variations of pH, temperature and DO concentration in CMC-xylan medium. 76

3.20 Batch fermentation in bioreactors: Variation of ATP in newspaper medium 77

3.21 Batch fermentation in bioreactors: Variation of ATP concentration in CMC-xylan medium. 78

3.22 Batch fermentation in bioreactors: Variation of biomass concentration in CMC-xylan medium. 79

3.23 Batch fermentation in bioreactors: Variation of ATP pool in CMC-xylan medium 81

3.24 Batch fermentation in bioreactors: Variation of xylanase activity in newspaper medium 82

3.25 Batch fermentation in bioreactors: Variation of xylanase activity in CMC-xylan medium 83

3.26 Batch fermentation in bioreactors: Variation of CMCase activity in newspaper medium 86

3.27 Batch fermentation in bioreactors: Variation of CMCase activity in CMC-xylan medium 87

3.28 Batch fermentation in bioreactors: Variation of FPase activity 90

3.29 Batch fermentation in bioreactors: Variation of protein concentration in newspaper medium. 92

3.30 Batch fermentation in bioreactors: Variation of protein concentration in CMC-xylan medium. 93

3.31 Batch fermentation in bioreactors: Variation of k_La and $Q_{O_2}C$ in newspaper medium 96

3.32 Batch fermentation in bioreactors: Variation of k_La and $Q_{O_2}C$ in CMC-xylan medium 97

3.33 Microflora in bioreactors immediately after inoculation. . . 100

3.34 Microflora in bioreactors on the first day 101

3.35 Microflora in bioreactors on the second day. 102

3.36 Microflora in bioreactors on the third day 103

3.37 Microflora in bioreactors on the fourth day. 104

3.38 Microflora in bioreactors on the fifth day 105

3.39 Conidia and conidiospores of the acidophilic fungus (1 000 x). 108

A1.1 Standard calibration curve for ATP 127

A2.1 Standard calibration curve for protein 129

A3.1 Standard calibration curve for glucose 131

CHAPTER 1

INTRODUCTION

1.1 DEFINITIONS

The term "cellulolytic enzymes" as employed hereinafter designates an enzyme complex capable of depolymerizing and hydrolyzing various cellulosics and lignocellulosics to oligosaccharides and ultimately to monosaccharides such as glucose (from cellulose) and xylose (from hemicellulose). The term "cellulase" as employed hereinafter designates an enzyme complex capable of depolymerizing and hydrolyzing cellulose into cellodextrins, cellobiose and glucose. The term "hemicellulase" as employed hereinafter designates an enzyme complex capable of depolymerizing hemicellulose into oligo-, di- and monosaccharides, mostly pentoses. Thus, the cellulolytic enzymes are comprised of cellulases and hemicellulases.

1.2 ASEPTIC AND STERILE CONDITIONS FOR ENZYME PRODUCTION AND FOR ENZYMATIC HYDROLYSIS

The production of liquid fuels such as ethanol from cellulosics/-lignocellulosics via enzymatic hydrolysis has not yet proven to be economically feasible. This is because of the high fraction of cost associated with the enzyme-production and saccharification steps.

i) Enzyme production: All known commercial and pilot scale processes, for the production of extracellular cellulolytic enzymes by fermentation of the defined microorganisms (in contrast to mixed flora of unidentified microorganisms) in liquid medium (in contrast to solid-

state substrate fermentation), have to be carried out under aseptic and sterile conditions using pharmaceutical standards for equipment and, also, for operating procedures. This constraint has resulted in capital investment and operating cost requirements which have been excessively high for the commercial production of cellulolytic enzymes. The high cost, associated with sterile and aseptic fermentation on the industrial level, is the major obstacle to the commercial production of cellulolytic enzymes as bulk chemicals or bulk biochemicals for other industrial uses. Examples of these uses are the processes for conversion of cellulosic feedstocks into fuel-grade or chemical-grade ethanol. Examples of pilot-scale processes are the Gulf-Arkansas University process (Emert et al. 1980; Anonymous 1982) and the Iotech-Natick process (Chahal 1982; Stone 1982).

ii) Enzymatic hydrolysis or saccharification: The presently-known cellulolytic enzymes are most active within the pH range of 5,0 to 7,0. This implies that the saccharification process for the enzymatic depolymerization and hydrolysis of cellulosics and lignocellulosics should be carried out within this pH range, at which all types of microbial contamination from a nonsterile environment are possible. In order to avoid or to minimize microbial contamination during saccharification and storage periods, either sterile conditions have to be applied or antibiotics used. An example is the work of Reese and Mandels 1980 which found that tetracycline and chlortetracycline are the best antibiotics for controlling microbial contamination during saccharification using the cellulolytic enzymes of Trichoderma reesei QM 9414.

During the last decade, research efforts have concentrated on the improvement of enzyme productivity and quality. The productivity/-quality of the enzymes have been significantly improved by optimizing physical/chemical conditions for fermentation; by creating new mutants such as the strains QM 9414 (Mandels et al. 1971, MCG 77 (Gallo et al.

1978), Rut C30 and NG 14 (Montenecourt et al. 1980), L-series (Shoemaker et al. 1983a), VTT-Series (Finland) (Nevalainen and Palva 1978), D-series (India) (Ghosh et al. 1982; Mishra et al. 1982) and the Iotech strains (Smith 1984) from the wild strain Trichoderma reesei QM 6a; and even by cloning the Trichoderma reesei cellulase genes into the fast growing bacterium Escherichia coli (Shoemaker et al. 1983b; Teeri et al. 1983). However, the requirement of sterile/aseptic operating conditions still remains as an economic disadvantage for large scale production.

1.3 SEARCH FOR NONASEPTIC AND NONSTERILE PROCESS

Fermentation processes could be operated under (i) aseptic and sterile conditions, such as in the case of all pharmaceutical processes; (ii) sanitary conditions, such as the case of dairy and brewing processes; and (iii) nonaseptic and nonsterile conditions, such as in the case of biological processes for wastewater treatment.

In the aseptic and sterile process, the fermenter and all equipment, accessories, materials, ingredients which are in direct contact with the culture medium have to be sterilized and germ-free. The inoculum used to seed the fermenter has to be a pure culture. All operation conditions have to be aseptic in order to avoid microbial contamination.

In the sanitary process, the fermenter and all equipment, accessories, materials, ingredients which are in direct contact with the culture medium have to be sterilized or disinfected. The inoculum used to seed the fermenter is preferably pure culture but is not required to be absolutely pure. All operating conditions are non-aseptic.

In the nonaseptic and nonsterile process, the microbial cultures and the fermentation are conducted in an open tank, container or pond.

The culture medium requires no sterilization and the operation requires no special precautions against microbial contamination.

A nonaseptic and nonsterile process for axenic or pure cultures does not exist. However, it is possible to develop a nonaseptic and nonsterile process for the cultivation or the fermentation of one single species (or single strain). It is based on the following principle: the selected microorganism used for the production of a desirable fermentation product has to be able to grow and to synthesize the product under extreme physiological conditions of pH, temperature, chemical environments, etc. Under such conditions, contamination by other species could be eliminated or reduced to an insignificant level. If the conditions and the microorganism are appropriately choosen, fermentation could be operated under nonaseptic and nonsterile condition with the desired strain, as in the case of the pure culture. Table 1.1 lists some axenic cultures potentially useful for the production of extracellular cellulolytic enzymes under nonaseptic and nonsterile conditions.

1.4 ACIDOPHILIC FUNGUS AND ACIDIC PROCESS FOR THE FERMENTATIVE PRODUCTION OF CELLULOLYTIC ENZYMES

Recently, we succeeded in developing a novel process for the production of extracellular cellulolytic enzymes by fermentation under nonaseptic and nonsterile conditions. It consists of carrying out this fermentation process under very acidic conditions of pH < 3 by using the appropriate strain of acidophilic/acid-resistant/acid-tolerant cellulolytic microorganism. Microbial contamination is thus avoided or minimized to an insignificant level under this acidic pH condition. This novel process has worked on the laboratory scale, in up to 12 ℓ of culture medium in 5-gallon carboys for a period of 10 days, by using the newly isolated acidophilic fungus strain NC-11 from our private (LeDuy) culture collection of acidophilic fungi. This strain NC-11 has been deposited at the American Type Culture Collection under accession

Table 1.1 - Axenic cultures potentially useful for the production of extracellular cellulolytic enzymes under nonaseptic and nonsterile conditions.

Microorganism	Enzyme production	Enzyme properties	References
THERMOPHILES:			
Thielavia terrestris	40 - 80°C pH 4,5 - 6,0	Crude CMCase and Avicelase Optimal temperature 65 - 80°C Optimal pH 5,5 - 8,0	Bellamy et al. 1974; Skinner and Tokuyama 1978.
Thermomonospora sp.	55°C pH 7,2	Crude CMCase and Avicelase Optimal temperature 70°C (CMCase) and 65°C (Avicelase) Optimal pH 5,9 (CMCase) and 7,0 (Avicelase)	Hagerdal et al. 1980; Moreira et al. 1981.
Clostridium thermocellum	60 - 64°C Anaerobic	Crude CMCase and Avicelase Optimal temperature 64 - 65°C Optimal pH 5,2 - 5,4	Ng et al. 1977.
ALKALOPHILES:			
Bacillus sp. N-4	pH 8 - 11 37°C	Partially purified CMCase Optimal pH 10 Optimal temperature 50°C	Horikoshi et al. 1984.
ACIDOPHILES:			
Acidophilic fungus NC-11	pH 2 28 - 30°C	Crude CMCase and xylanase Optimal pH 3 Optimal temperature 55°C (CMCase) and 25 - 50° (xylanase)	Cauchon 1984; Cauchon and LeDuy 1984, 1985.

number ATCC-20677 for patent application purposes. A patent application has been filed for this process. We believe that the discovery of the acidophilic cellulolytic fungi, together, with the invention of the acidic fermentation process, could have a significant impact on commercial applications owing to the fact that aseptic/sterile conditions are not required for full-scale operations. All scientific and technical details on the process and the products are reported elsewhere (Cauchon 1984; Cauchon and LeDuy 1984, 1985).

The most relevant features of this novel process are the following:

1) This method of fermentative production of extracellular cellulolytic enzymes under nonaseptic and nonsterile conditions requires very low capital investment and operating costs for industrial-scale application. Fermentation can be conducted in an open tank, container or pond with a plastic lining instead of a very expensive stainless steel pharmaceutical aseptic fermenter. The culture medium requires no sterilization and the operation requires no special precautions against microbial contamination.

2) Acidophilic fungus strain NC-11 grows and produces at least two enzymes of the extracellular cellulolytic enzymes, carboxymethylcellulase (CMCase) and xylanase in a culture medium at pH 2,0 under nonsterile and nonaseptic conditions. The filter paper (FPase) volumetric activity (IU) and specific activity (IU/mg protein) of the Trichoderma reesei Rut C-30 culture filtrate (which was prepared in shaken flasks at the laboratory of Dr. A.W. Khan, NRC, Ottawa) are 100 times and six times those of the acidophilic fungus culture filtrate, respectively.

3) The culture medium for this production consists of a carbon source from cellulosics/lignocellulosics, such as Na-CMC, xylan, Avicel cellulose, cellulose powder, α-cellulose, sawdust, newsprints, etc. or

a mixture of the a forementioned, together with simple ingredients, such as $(NH_4)_2SO_4$, K_2HPO_4, and traces of $MgSO_4$ and $NaNO_3$. The pH is adjusted to 2,0 with a mineral acid such as H_2SO_4. Fermentation is carried out at room temperature, preferably at 28-30 °C, under aerobic conditions and without control of the pH. Two economic advantages are thus accounted for: the requirement of simple and inexpensive ingredients and the lack of a need to control the pH due to the self- stabilization of the culture medium pH during the fermentation process.

4) The CMCase and xylanase produced from the acidophilic fungus strain NC-11 are stable under very simple storage conditions, such as in the fresh culture medium without substrate for a period of three days at any temperature from 0 to 30 °C. These extracellular enzymes have an optimum pH around 3,0, with the best range of pH from 2,0 to 3,6 for any temperature between 15 and 60 °C. The optimum temperatures are 55 °C for CMCase activity and 25 to 50 °C for xylanase activity, for any pH between 2,0 and 5,2. Thus, the extracellular cellulolytic enzymes of the acidophilic fungus strain NC-11 are entirely different from other presently-known cellulolytic enzymes owing to NC-11's optimum acidic pH range from 2,0 to 3,6 for enzyme activities.

5) This implies that the saccharification process or the depolymerization and hydrolysis of cellulosics/lignocellulosics can be operated under acidic conditions, such as at pH 2 or 3 for the best enzyme activities, at the same time controlling microbial contamination. Thus, this process offers further economic advantages for industrial applications because the extracellular cellulolytic enzymes can be stored and used under nonaseptic and nonsterile conditions.

6) In addition, when the cellulosics/lignocellulosics are pretreated with any acidic process prior to the saccharification step, neutralization of the pretreated slurries can be avoided or minimized

to a very low cost. This can be achieved by utilizing the extracellular cellulolytic enzymes for saccharification; thereby, saccharification proceeds under acidic conditions.

1.5 R&D STRATEGY AND GENERAL OBJECTIVE

Having the acidophilic cellulolytic fungus strain NC-11 on hand, and having proven that the fermentative production of the extracellular cellulolytic enzymes can be conducted under nonaseptic and nonsterile conditions in laboratory scale experiments, as shown by stage A in Figure 1.1, does not mean that the pilot scale testing experiments, as shown by stage H in Figure 1.1, could be carried out immediately. On the contrary, between discovery in the laboratory and pilot scale testings, there are many stages of R&D which need to be completed in order to ensure successful pilot scale experiments. The strategy for some essential R&D stages (stages B to G) are schematically shown in Figure 1.1.

The next logical step is the improvement of enzyme productivities by: (i) creating the mutants from the wild strain NC-11 using classical physical/chemical methods, (ii) searching for an economical chemical inducer of cellulolytic enzymes, and (iii) optimizing fermentation conditions using kinetic data. This is referred to as stage B in Figure 1.1. Paralleling stage B, a project on the isolation, purification and characterization of the strain NC-11 cellulolytic enzymes could be carried out. This is referred to as stage C in Figure 1.1. If the improvement of the enzyme productivities from stage B is significant, the preliminary design for pilot plant testings (stage E in Figure 1.1) could be started. The findings from stage B will dictate the design of the bioreactor (depending on the oxygen requirements for growth and for enzyme productions) and the choice of fermentation type (cell-free, immobilized cells, or solid-state substrate, depending on growth- or nongrowth-associated enzyme productions) in stage E. If the improvement of enzyme productivities in stage B is insignificant,

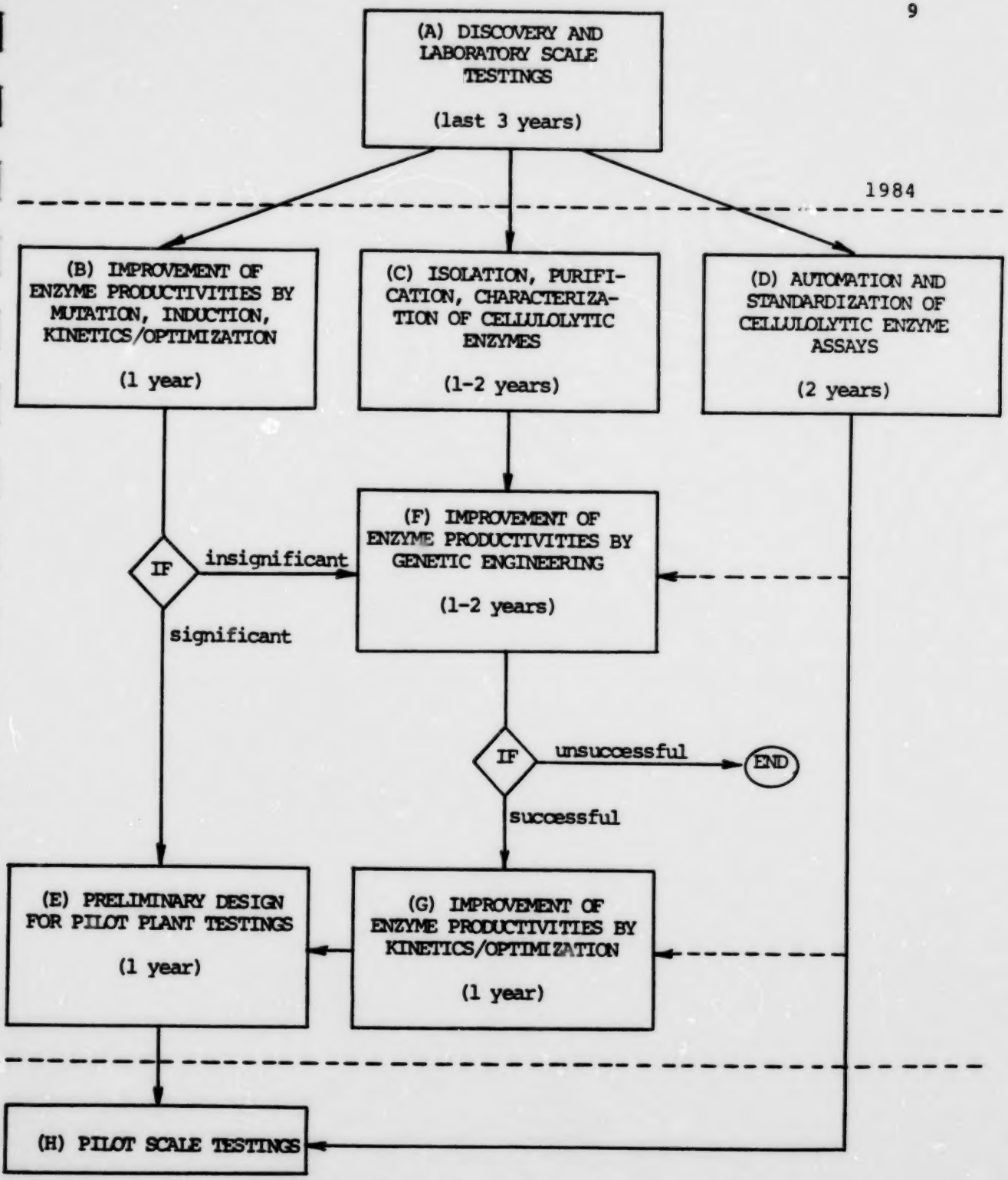


Fig. 1.1 - Strategy for process R & D.

genetic engineering improvement methods should be considered (stage F in Figure 1.1). These consist of isolating the messenger RNA(s) of the acidophilic fungus NC-11 strain by the antibody method, synthesis of the gene(s) through reverse transcription, and cloning of these genes into fast growing acidophilic/acid-resistant/acid-tolerant bacterium. If genetic improvement of enzyme productivities is unsuccessful, which is most unlikely, the whole project will be stopped. If successful, productivities of the cellulolytic enzymes from genetically modified bacteria could be further improved by optimizing fermentation conditions from kinetic data. This is refered to as stage G in Figure 1.1. Another project, on the automation and standardization of the cellulolytic enzyme assays, could be carried out simultaneously with stages B and C (stage D in Figure 1.1). This automated instrumental device would be extremely useful for process monitoring and control, and for quality control during pilot scale testings (stage H in Figure 1.1), and also as the measuring instrument for any R&D stage shown in Figure 1.1.

The object of this project is to investigate stage B in Figure 1.1 only - that is, to attempt to improve enzyme productivities by creating new mutants, by searching for an economical chemical inducer, and by optimizing fermentation conditions in terms of oxygen supply.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Microorganism

The acidophilic cellulolytic fungus strain NC-11, which has been deposited in the American Type Culture Collection as "Patent Culture ATCC-20677", was used in this study (Figure 2.1). The culture was maintained by periodic transfers, at room temperature, in a liquid medium containing (w/v %): glucose 1,0; $(NH_4)_2SO_4$ 0,1; K_2HPO_4 0,1; $NaNO_3$ 0,1; $MgSO_4.7H_2O$ 0.016. The initial pH of the culture medium was adjusted to 2,0 with H_2SO_4 after being autoclaved.

2.1.2 Chemicals

Table 2.1 lists and specifies all chemicals used in this project.

2.1.3 Bioreactor system

Five open-pond 10 ℓ rectangular bioreactors were designed and built by us. The bioreactors were made of Plexiglas and equipped with a stainless steel coil heat exchanger for temperature control. A cover in Plexiglas with a built-in condenser was provided for minimizing medium loss due to aeration. The geometry of the bioreactors was designed in such a way that no mechanical agitation is required for a good mixing of the culture media even when solid substrates such as saw

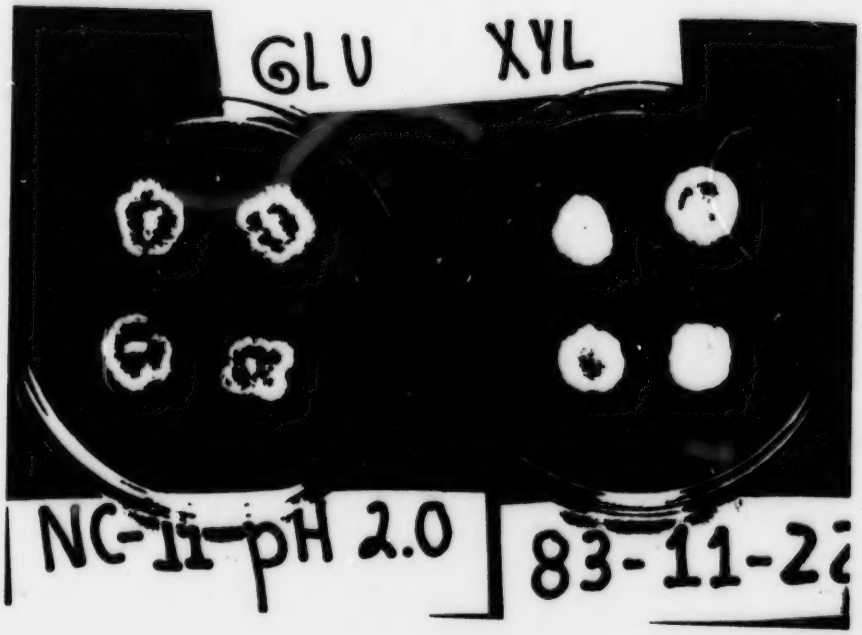


Fig. 2.1 - Pure culture of the acidophilic fungus NC-11 (ATCC-20677) grown in glucose and xylose at pH 2,0.

Table 2.1 - Chemicals used.

Chemicals	Trade name	Remarks
FOR CULTURE MEDIA:		
D(+) Glucose	Fisher	
D(+) Sucrose	Fisher	
D(+) Cellobiose	Sigma	
D(+) Maltose	BDH	
D(-) Fructose	Baker	
D(+) Galactose	Baker	
Lactose	Anachemia	
D(+) Xylose	Sigma	
Starch	Merck	
Avicel cellulose PH-101	FMC	microcrystalline
Cellulose powder CC-31	Whatman	microgranular
α-Cellulose	Sigma	
Na-Carboxymethylcellulose (Na-CMC)	Sigma	low viscosity
Xylan	Sigma	from larchwood
Newspaper		ball-milled
Newsprint	Abitibi Paper Co.	homogenized
Cardboard carton		homogenized
Sawdust		from softwood
Panomalt Regular Syrup	Fleischmann	contains 55 to 63% maltose; 76,8% solids and 3,8% protein

Table 2.1 - Chemicals used (continued).

Chemicals	Trade name	Remarks
Malt extract (ME)	Difco	
Malt extract agar (MEA)	Difco	
Agon	Difco	
Yeast-malt broth (YMB)	Difco	
$(NH_4)_2SO_4$	Anachemia	
K_2HPO_4	Fisher	
$NaNO_3$	Fisher	
$MgSO_4 . 7H_2O$	Anachemia	
H_2SO_4	BDH	
Antifoam FG-10	Dow Corning	food grade
FOR MUTATION:		
Ethyl methanesulfonate (EMS) = Methane sulfonic acid ethyl ester	Sigma	
$NaNO_3$	Baker	
N-Methyl-N'-nitro-N-nitrosoguanidine (NTG)	Sigma	
FOR ANALYTICAL REAGENTS:		
Tris(hydroxymethyl)aminomethane	Ultrol (Calbiochem)	
Luciferine-luciferase FLE-50	Sigma	

Table 2.1 - Chemicals used (continued).

Chemicals	Trade name	Remarks
Adenosine triphosphate (ATP)	Sigma	
Congo Red	Sigma	
Remazol Brilliant Blue R.	Sigma	sodium salt
Sodium sulfate	Anachemia	
Sodium phosphate tribasic	Fisher	
Methanol	BDH	
3,5-Dinitrosalycilic acid	Sigma	crystalline
Sodium sulfite	Anachemia	anhydrous
Phenol	Anachemia	loose crystals (99,1% pur)
Filter paper	Whatman	no 1 chromatography
Bovine albumin	Sigma	
Trichloroacetic acid (TCA)	Fisher	
Na_2CO_3	Anachemia	
$CuSO_4 . 5H_2O$	Baker	
Na-tartrate . $2H_2O$	Anachemia	
Phenol reagent	BDH	folin-ciocalteau
NaOH	Anachemia	pellets
Ether	Canlab	purified
Ethanol 95%		
Acetic acid	Caledon Laboratories Ltd.	glacial
Sodium acetate	Fisher	fused-anhydrous

dust, ball-milled newspaper, etc. were used. The mixing of the culture media was done solely by aeration. The dimensions and the details of the bioreactor are shown in Figures 2.2 and 2.3.

A temperature-controlled water bath with circulating pump was installed for controlling the temperature of the bioreactor system. The aeration rate was controlled by air flowmeters. Control of the pH of the culture media during fermentation was not required. The bioreactor system is shown in Figure 2.4.

2.2 EXPERIMENTAL METHODS

2.2.1 Strategic plan

The experiment's strategic plan shown in Figure 2.5 consists of a series of task descriptions and the chronological decision-making guideline.

TASK (a): To produce cellulolytic enzymes (FPase activity) by growing the acidophilic fungus on various carbohydrate substrates. Research staff B; 3 months.

A large number of carbohydrate substrates will be used for the tests (such as glucose, fructose, xylose, maltose, lactose, sucrose, cellobiose, Avicel cellulose, cellulose powder, α-cellulose, Na-CMC, xylan, newspaper, newsprint, sawdust, cardboard carton, etc.) The experiments will be conducted at pH 2,0 and at 30 °C and under nonsterile/nonaseptic conditions. This series of experiments will serve as control experiments using the wild strain of acidophilic fungus. The results obtained will dictate the choice of the substrates to be used for tasks (b), (c) and (d).

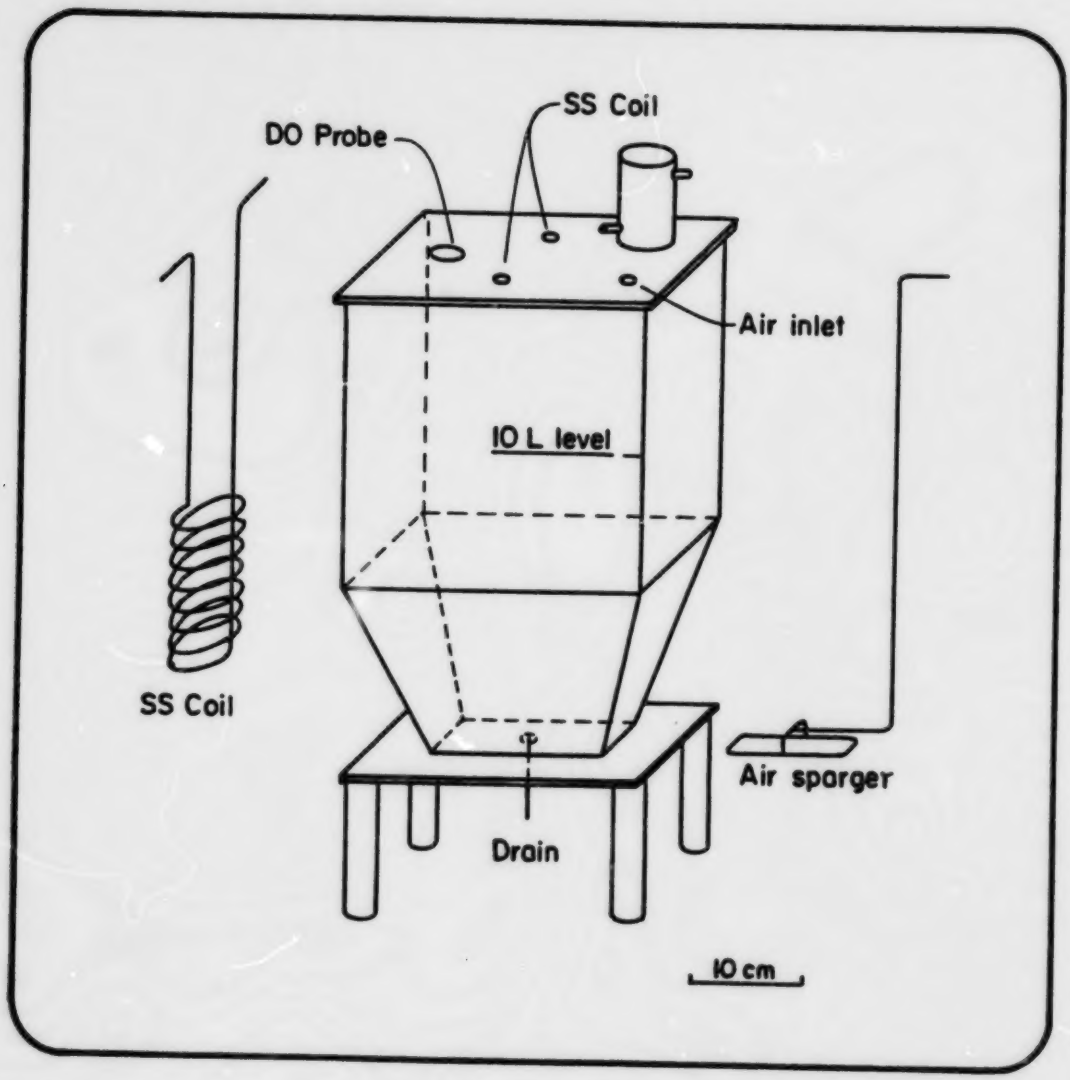


Fig. 2.2 - Detail of the bioreactor.

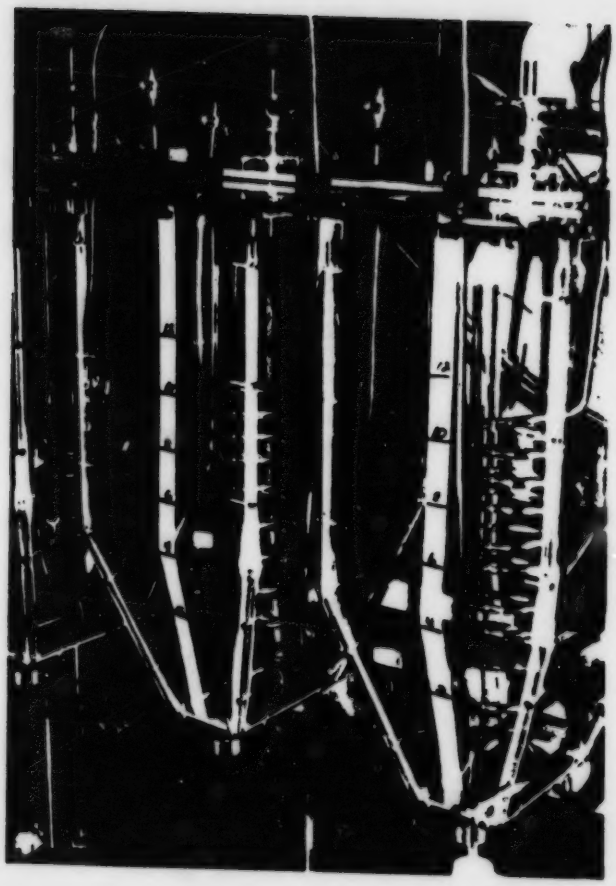


Fig. 2.3 - Detail of the bioreactor.

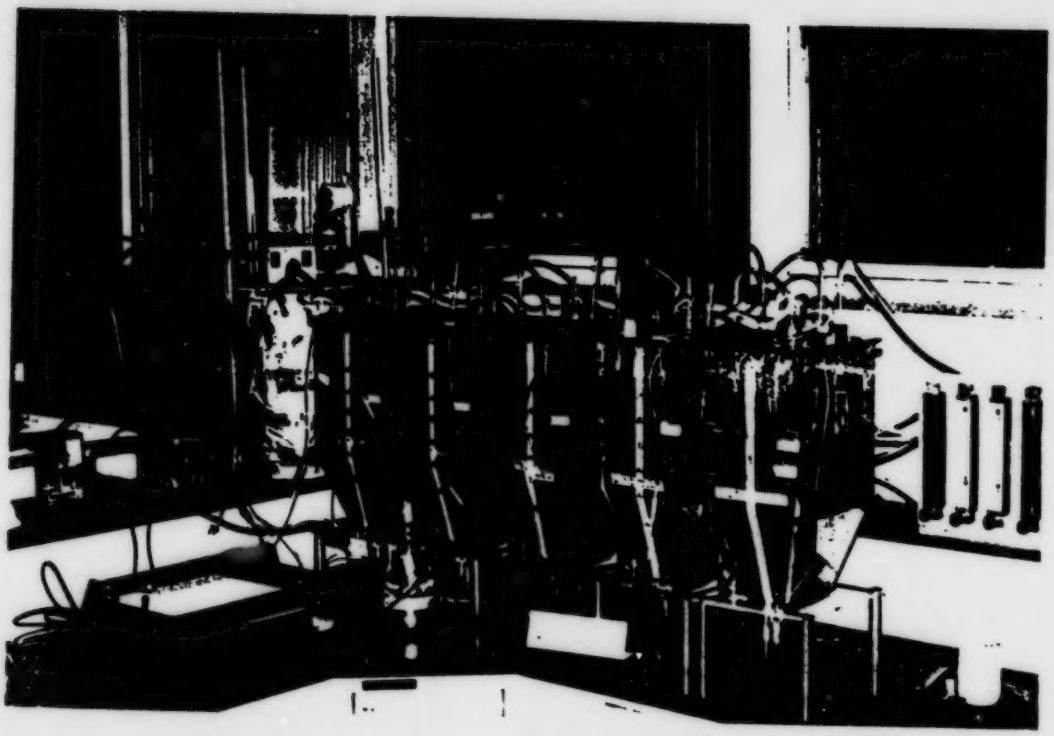


Fig. 2.4 - Bioreactor system.

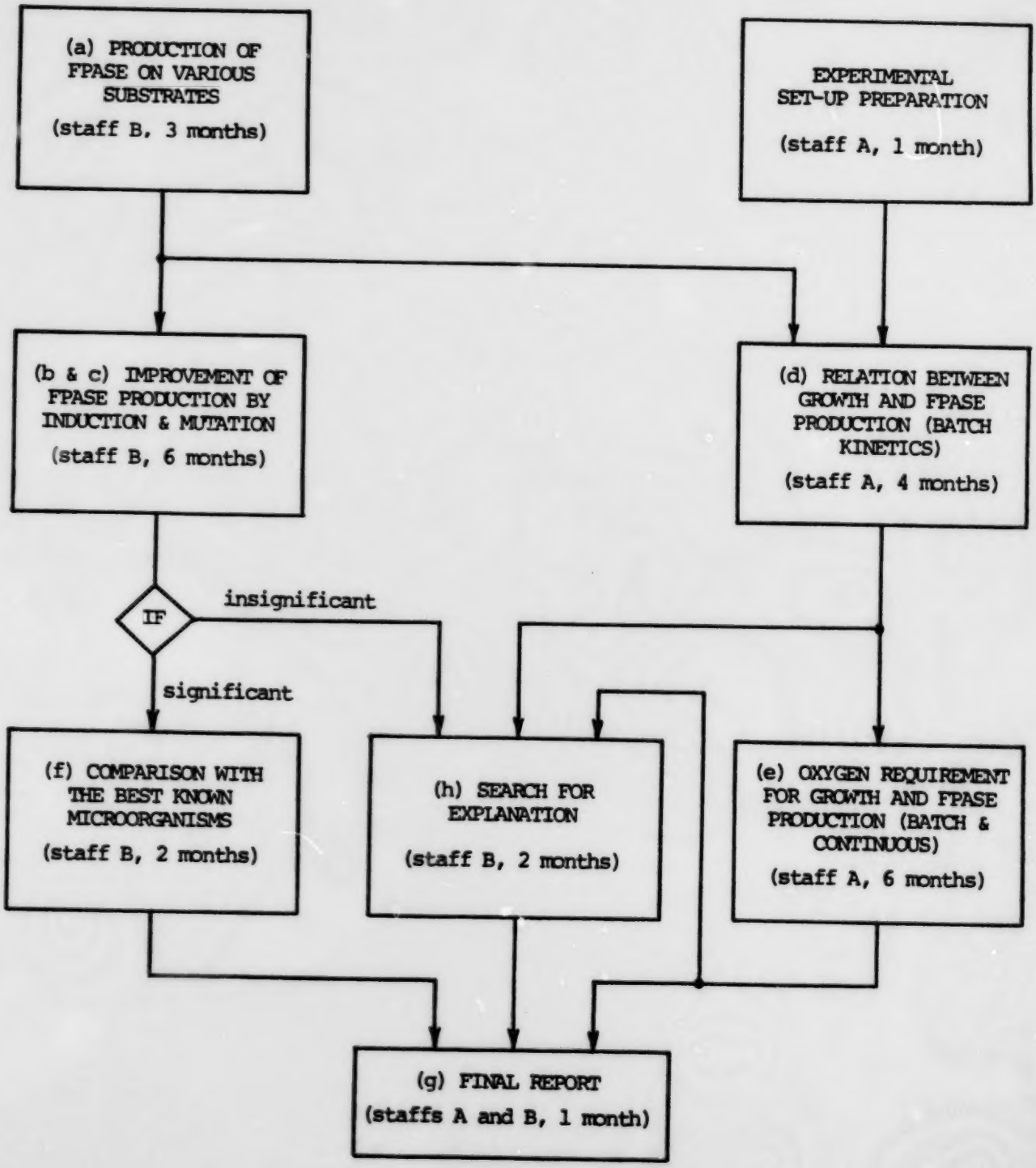

(a) PRODUCTION OF FPASE ON VARIOUS SUBSTRATES
(staff B, 3 months)
EXPERIMENTAL SET-UP PREPARATION
(staff A, 1 month)
(b & c) IMPROVEMENT OF FPASE PRODUCTION BY INDUCTION & MUTATION
(staff B, 6 months)
(d) RELATION BETWEEN GROWTH AND FPASE PRODUCTION (BATCH KINETICS)
(staff A, 4 months)
IF
insignificant
significant
(f) COMPARISON WITH THE BEST KNOWN MICROORGANISMS
(staff B, 2 months)
(h) SEARCH FOR EXPLANATION
(staff B, 2 months)
(e) OXYGEN REQUIREMENT FOR GROWTH AND FPASE PRODUCTION (BATCH & CONTINUOUS)
(staff A, 6 months)
(g) FINAL REPORT
(staffs A and B, 1 month)

Fig. 2.5 - Experiment strategic plan.

TASKS (b) and (c): To attempt enhancing the production of the FPase by induction with various chemical inducers and by mutations with various physical and chemical methods. Research staff B; 6 months.

It is well known that the production of cellulolytic enzymes could be greatly enhanced by using various chemical inducers (Rho et al. 1982; Sternberg and Mandels 1979, 1980). We will search for the best chemical inducer for the production of cellulolytic enzymes under non-sterile and nonsapetic conditions with the acidophilic fungus NC-11. The ratio of the extra cost of the inducer to gained enzyme productivity will be used as the selection criterion for choosing the best inducer for industrial application. From this standpoint, it is a clear priority that the best known chemical inducers such as sophorose (Sternberg and Mandels 1979, 1980) and thiocellobiose (Rho et al. 1982) will not be selected due to their excessively high cost. Many carbohydrates and non-carbohydrate chemicals, especially those available as by-products of wastes, will be tested according to the procedures already established by other researchers (Rho et al. 1982).

High-yield strains of Trichoderma reesei such as Natick MCG77, Rut-C30, Czechoslovakia MHC-15 and MHC-22, etc. have been obtained from the low-yield wild strain Natick QM 6a by applying classical physical and chemical mutation methods (UV-radiation, nitrosoguanidine) by other researchers (Montenecourt et al. 1980; Labudova et al. 1981; Tangnu et al. 1981). We will attempt to create new mutants from the wild strain of acidophilic fungus NC-11 by using such classical methods. The selection criterion will be the high yield of FPase activity. Prior to the experiments on mutations, a semi-quantitative method for detecting the positive mutants has to be established. This method will be developped based on the dyed substrate method (Smith 1977), the congo red indicator method (Teather and Wood 1982), and the fine cellulose substrate method (Nummi et al. 1981).

If improvement of FPase productivity by induction and by mutation methods from tasks (b) and (c) is significant, the study will proceed to task (f). If no improvement results or the improvement obtained is insignificant, the study will lead to task (h).

TASK (f): To compare the best results obtained from tasks (b) and (c) with other best known organisms, such as the mutant strains of Trichoderma reesei, or the like, in terms of FPase production and the production of other celluloytic enzymes such as CMCase, Avicelase, xylanase, and β-glucosidase. Research staff B; 2 months.

This comparative study will be based mainly on available data. If collaboration from other laboratories are available, several short experiments on the fermentative production of extracellular cellulolytic enzymes from their "star" strains will be carried out to collect the desired data for comparison.

TASK (d): To ascertain whether the production of FPase is growth-associated or non-growth associated, or both. Research staff A; 4 months.

This is a study of the kinetics of batch fermentation with acidophilic fungus for the production of cellulolytic enzymes. One or two of the most interesting substrates, determined from the results in task (a), will be used for kinetic studies. The experiments will be conducted under nonaseptic and nonsterile conditions, at pH 2,0 and at 30 °C, in bioreactors containing at least 10 ℓ of culture medium each. Conventional type bioreactors will be made of plexiglass or plastic, with devices for aeration/agitation control. During the experiments, the biomass concentration of C (in terms of ATP, or another equivalent variable) and the concentration of cellulolytic enzymes P will be monitored. From is data:

(i) we will be able to determine if the production of cellulolytic enzymes is growth-associated, nongrowth-associated, or both,

$$\frac{dP}{dt} = a\left(\frac{dC}{dt}\right) + b\,(C) \qquad (2.1)$$

This kinetics model has been sucessfully used for lactic acid fermentation (Luedeking and Piret 1959) and for xanthan gum fermentation (Weiss and Ollis 1980). Knowledge of the relationship between growth and product formation will be essentiel for making decisions concerning the design of bioreactors for pilot scale experiments.

(ii) we will check the correlation between the FPase and other variables. This correlation, if found, will be very useful as an indirect way of monitoring FPase in the culture medium.

TASK (e): To determine the oxygen requirements in terms of specific respiration rate Q_{O_2} and dissolved oxygen concentratin DO for the growth and the production of FPase. Research staff A; 6 months.

This is a study of the kinetics of batch and continuous fermentations for the production of cellulolytic enzymes with emphasis on the oxygen requirements. Oxygen requirements will be analyzed in terms of critical dissolved oxygen concentration (DOcrit), the oxygen transfer coefficient (k_La), and the specific respiration rate (Q_{O_2}) for both cell growth and enzyme production, in batch, as well as in continuous fermentations. The dissolved oxygen concentration will be monitored with DO-meters. The k_La and Q_{O_2} will be determined with the dynamic gassing-out method (Atkinson and Mavituna 1983). The maintenance coefficient for oxygen (Mo) for cells will be determined from continuous data using the method developed by other researchers (Ryu et al. 1979).

The following information will be expected to be gained from this task: the relationship between Q_{O_2} and specific growth rate μ as well as specific enzyme production rate ν; critical oxygen concentrations for cell growth and enzyme production; and the maintenance coefficient for oxygen Mo. These are essential for the preliminary design of the pilot scale process.

TASK (h): Search for an explanation should tasks (b) and (c) fail. Research staff B, 2 months.

This study will be based on: the examination and synthesis of data obtained from other tasks (a) to (e); the locating of information gained from previous research; and if possible, consultation/advice from other laboratories.

2.2.2 Enzyme production from various substrates

The production of extracellular cellulolytic enzymes (CMCase, xylanase, FPase) from various carbohydrate substrates, under nonaseptic and nonsterile conditions, was conducted in 1-ℓ Erlenmeyer flasks containing 500 mℓ of culture medium each. The following carbohydrate substrates were used at a concentration of 1% (w/v): glucose; sucrose; cellobiose; maltose; fructose; galactose; lactose; xylose; starch; Avicel cellulose; cellulose powder; α-cellulose; Na-CMC; xylan; newspaper; newsprint; sawdust; cardboard carton; and a mixture of Na-CMC and xylan each at 1% (w/v). The newspaper, newprint, sawdust and cardboard cartons were homogenized to fine fibers with a Model PT 10-35 Polytron Homogenizer (Brinkmann Instruments) using a Model PT 20 ST Saw-Tooth Generator. The basal medium contained in w/v %: $(NH_4)_2SO_4$ 0,1; K_2HPO_4 0,1; $MgSO_4.7H_2O$ 0,016; and $NaNO_3$ 0,1. The initial pH was adjusted to 2,0 with H_2SO_4. Five-day old inoculum previously grown in Yeast Malt Broth (Difco) under nonaseptic and nonsterile conditions was

used to inoculate the Erlenmeyers at 5% (v/v). Prior to inoculating the Erlenmeyers, the inoculum was washed five times with a basal salt solution in order to remove all enzymes and/or reducing sugars adhering to the mycelium. The experiment was conducted on a shaker at 150 rpm and at 30 °C for 14 days (Figure 2.6). The culture media were not sterilized and the experiment was carried out under nonaseptic condition. Samples were taken at 0, 1, 7 and 14 days for assays of extracellular cellulolytic enzymes (xylanase, CMCase, FPase).

2.2.3 Induction

The mycelial cells used for the induction tests were prepared by growing the acidophilic fungus in a glucose medium described in paragraph 2.1.1. Approximately 6 g of biomass obtained from 6 ℓ of medium were washed twice with a basal salt solution at pH 2,0 (e.g. culture medium without substrate). The remaining 3-4 g of washed cells were then resuspended in 500 mℓ of basal salt solution at pH 2,0 for induction tests.

The procedure for induction was based on the one reported by Rho et al. 1982. A 50 mℓ sample of the appropriate inducer prepared in the same basal salt solution at pH 2,0 was added to 50 mℓ of cell suspension in seven 250 mℓ Erlenmeyer flasks containing approximately 100 5 mm-glass beads. The flasks were incubated at 30 °C on a shaker at 150 rpm for various lengths of time. Samples for measuring enzyme activities (xylanase, CMCase and FPase) (15 mℓ) were taken every hour during the first 12 hours and one sample was taken at the 24th hour. Samples were alternately taken in seven Erlenmeyers so that only 30 mℓ of culture medium were withdrawn from each Erlenmeyer flask.

The following carbon sources were tested for their inducing power, at 1% w/v under induction tests: Na-CMC, xylan, ball-milled newspaper,

Fig. 2.6 - Experiments on the production of enzymes on various substrates.

and sawdust (from softwood). Cellobiose was also tested for induction power at a concentrtion of 1 mM.

2.2.4 Mutation

Mutation experiments were done on fungal spores using physical methods (UV radiation; X-ray radiation) and chemical methods (ethyl methanesulfonate; N-methyl-N'-nitro-N-nitroguanidine; nitrous acide). Acidophilic fungus spores were produced on a petri dish containing Malt Extract Agar (MEA)-agar (2% w/v) medium. The three to four day-old petri dish was rinsed twice with 5 mℓ portions of basal salt solution at pH 2,0 for collecting the spores. The collected spore solution was immediately diluted 100 times for routine use in all mutation experiments. This final solution will be referred to hereafter as the spore suspension.

2.2.4.1 UV radiation

The procedure for UV radiation was developed based on the one reported by Eriksson and Johnsrud 1983. Portions of 2 mℓ of the spore suspension in watch glasses were exposed to the UV source consisting of two Westinghouse G30T6 Sterilamp of 45 W each, for various lengths of time. The distance between the samples and the UV source was 45 cm. Sterile aluminum foil was used as a UV-ray reflector during spore exposure. Portions of 0,25 mℓ of exposed spore suspension in a watch glass at 0, 0,5, 1, 2, 3, and 4 hours were uniformly plated on petri dishes containing MEA-agar medium. The petri dishes were incubated at 30 °C in darkness for 3 to 4 days. Whole survival colonies were removed from the petri dishes to test their capacity for producing extracellular FPase enzyme activity individually.

2.2.4.2 X-ray radiation

Portions of 2 mℓ of the spore suspension in the containers (X-cell Expandable X-Ray Liquid Cell) were exposed to the X-ray source (Philips Model PW 1540, 20 mA, 41 kV, tungsten source) for various lengths of time. Quantities of 0,25 mℓ of exposed spore suspension in the containers at 0, 2, 10, 30, 60, and 135 minutes were uniformly plated on petri dishes containing MEA-agar medium. The petri dishes were incubated at 30 °C for 3 to 4 days. Whole survival colonies were removed from the petri dishes to test their capacity for producing extracellular FPase enzyme activity individually.

2.2.4.3 Mutagens

The procedure for mutation test using ethyl methanesulfonate (EMS) was based on the one reported by Carlton and Brown 1981. Five mℓ portions of spore suspension were added to the test tubes containing 5 mℓ of EMS solution (0,1 mℓ of EMS diluted in 2,5 mℓ of basal salt solution at pH 2,0). The tubes were then incubated on a shaker at 150 rpm and at 30 °C for 0, 0,5, 1, 2, 3, and 4 hours respectively.

The procedure for mutation test using N-methyl-N'-nitro-N- nitrosoguanidine (NTG) was developed based on the one described by Carlton and Brown 1981 and Avalos et al. 1985. Nine mℓ portions of spore suspension were added to test tubes containing 1 mℓ of NTG solution (10 mg of NTG dissolved in 10 mℓ of basal salt solution at pH 2,0). The tubes were then incubated on a shaker at 150 rpm and at 30 °C for 0, 15, 30, 45, 60 and 75 minutes respectively.

The procedure for mutation tests using nitrous acid was developed based on the one reported by Carlton and Brown 1981. Portions of 9,85 mℓ of spore suspensions were added to test tubes containing 0,15 mℓ of spores suspensions were added to the test tubes containing 0,15 mℓ of fresh nitrous acid (0,2 M of $NaNO_2$ in basal salt solution at pH 2,0).

The tubes were then incubated on a shaker at 150 rpm and at 30 °C for 0, 0,5, 1, 2, 3 and 4 hours respectively.

After treatment with each mutagen, 0,25 mℓ of treated spore suspensions in the tubes were uniformly plated on petri dishes containing MEA-agar medium. The petri dishes were incubated at 30 °C for 3 to 4 days. Whole survival colonies were removed from the petri dishes to test their capacity for producing extracellular FPase activity individually.

2.2.4.4 Screening test for survival colonies

Whole survival colonies obtained from the petri dishes were individually put in a screwed-cap test tube (Kimax 16 x 150 mm) containing 6 mℓ of basal salt solution at pH 2,0 and one 1 x 6 cm strip of dyed paper. The tubes were incubated at 30 °C on a shaker at 150 rpm and in a horizontal position for one week. The culture filtrates were then analyzed for FPase activity and for realeased color of dye at 595 nm with a Model Spectronic 710 Bausch & Lomb Spectrophotometer. An incubated tube containing dyed paper in basal salt solution at pH 2,0 was used as a blank.

The method for the preparation of dyed paper was the one developed by Poincelot and Day 1972. About two dozen strips of Whatman No. 1 chromatographic paper (1 cm x 50 cm) were placed in an Pyrex tray (54 x 48 x 8 cm). The tray was preheated in a water bath at approximately 80 °C. Distilled water (250 mℓ, 100 °C) was gently poured on the tray with care in order to avoid air bubble formation. Remazol Brilliant Blue R (Sigma), 1,5 g in 250 mℓ of distilled water (110 °C) was then poured evenly on the paper strips. The dye was distributed by tipping the tray, causing the dye to swirl over the paper. At two-minute intervals for 10 minutes, 30,0 g of sodium sulfate in 100 mℓ distilled water (100 °C) was added to the tray in 20 mℓ portions. During this time, the tray was shaken continuously. Then 1,5 g of trisodium

phosphate in 15 mℓ of water (100 °C) was evenly dropped into the solution, mixed well, and allowed to remain for 10 minutes. The fluid was discarded, and excess dye was washed-off with distilled water (paper in tray during washing). When the rinse water was clear, the paper was rinsed twice with methanol and air dried. The dry-dyed paper was the cut into 6 cm length (approximately 50 mg each) strips .

2.2.5 Batch fermentation

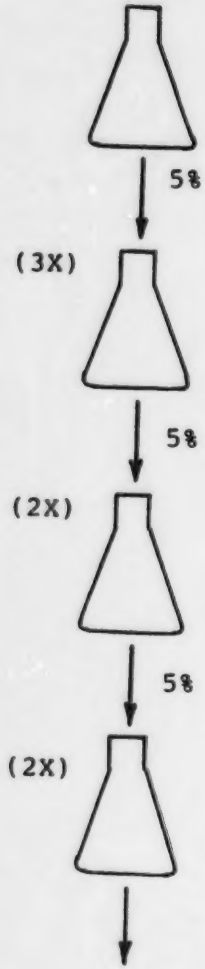
2.2.5.1 Inoculum preparation

Detailed information on the procedure for inoculum preparation is shown in Figure 2.7. All culture media were prepared with tap water, using the indicated ingredients, then sterilized in an autoclave at 121 °C for 20 minutes (except for two hours with 5-gallon carboys used in the transfer No. 6; Figure 2.7). The culture media were then cool down to room temperature and adjusted to pH 2 with concentrated H_2SO_4. All operations during the six transfers shown in Figure 2.7 were conducted under sterile and aseptic conditions.

Cells grown in two 5-gallon carboys (transfer No. 6) were harvested by gravity filtration through a synthetic fine tissue cloth. After the first filtration, the resulted 1,8 ℓ of cell paste containing 120 g/ℓ of biomass (on dry weight basis) were suspended and washed with 1 ℓ of basal salt solution (as medium used for the transfer No. 1, but without glucose; Figure 2.7) at pH 2, then filtered again through a fine tissue cloth. The washing and filtration was repeated four other times. At the end of the fifth filtration, the resulting 0,8 ℓ of cell paste was diluted to a total volume of 2 ℓ with basal salt solution at pH 2. The cell slurry was kept in the refrigerator (0-5 °C) for four days before inoculating the bioreactors.

The harvesting and washing operations were carried under nonsterile and nonaseptic conditions. It is to be noted that most of the

Fig. 2.7 - Batch fermentation in bioreactors : Inoculum preparation



Stock culture
glucose medium; pH 2; pure culture

5% (v/v)

(3X) Transfer 1

100 mL/250 mL
150 rpm
29-30°C
7d

Glucose	1%
$(NH_4)_2SO_4$	0,1%
K_2HPO_4	0,1%
$NaNO_3$	0,1%
$MgSO_4.7H_2O$	0,016%

5% (v/v)

(2X) Transfer 2

600 mL/1 L
150 rpm
29-30°C
13d

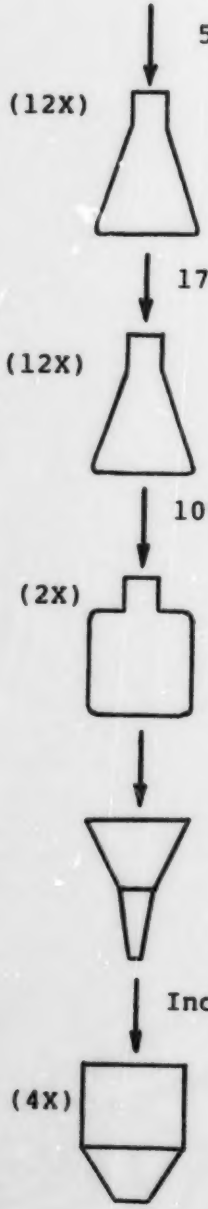
idem.

5% (v/v)

(2X) Transfer 3

idem.

idem.



Transfer 4

100 mL/250 mL
150 rpm
29-30°C
3d

ME	1,44%
$(NH_4)_2SO_4$	0,12%
K_2HPO_4	0,12%
$NaNO_3$	0,12%
$MgSO_4.7H_2O$	0,02%

Transfer 5

500 mL/1 L
150 rpm
29-30°C
5d

idem.

Transfer 6

16 L/5 gallon
0,22 vvm
27-28°C
12d

Maltose (Panomalt syrup)	0,9-1,0%
$(NH_4)_2SO_4$	0,1%
K_2HPO_4	0,1%
$NaNO_3$	0,1%
$MgSO_4 . 7H_2O$	0,016%

Harvesting & Washing

Batch fermentation

cells were recovered by filtration through a fine tissue cloth alone. Previous attempts to separate cells from culture medium by ultrafiltration using the Millipore 2GPM Pellicon Casette System and 0,464 m^2 (5 ft^2) Casette of 0,45 µm membrane were failures. The membrane pores were immediately plugged by the fungal cells.

2.2.5.2 Fermentation experiment

Batch fermentation experiments in bioreactors were conducted in duplicata in the 10 ℓ working volume bioreactors, under nonsterile and nonaseptic conditions. The culture medium contained either ball-milled newspaper at 1% w/v or Na-CMC low viscosity (Sigma)-xylan from larchwood (Sigma) at 0,2% w/v each, as carbon sources. Other ingredients were: K_2HPO_4 0,1%; $(NH_4)_2SO_4$ 0,1%; $NaNO_3$ 0,1% and $MgSO_4.7H_2O$ 0,016%. Initial pH was adjusted to 2 with concentrated H_2SO_4. The media were inoculated with 9% v/v of inoculum previously prepared (see paragraph 2.2.5.1). The fermentation experiments were conducted at ~27 °C for 10 days, at an aeration rate of 0,35 vvm for CMC-xylan medium and 0,92 vvm for newspaper medium.

Samples (25 mℓ portions) were periodically taken from bioreactors to measure pH, biomass concentration, ATP concentration, protein concentration, and cellulolytic enzyme activities (xylanase, CMCase and FPase). Direct measurements of DO concentration, K_La and Q_{O_2} were also periodically done. Daily observation of the contamination level in the bioreactors was done by a streak-plate technique on petri dishes.

2.2.6 Continuous fermentation

Continuous fermentation of the acidophilic fungus was conducted with the same bioreactor system used previously for batch fermentation experiments. Two Nalgel tanks containing 150 ℓ each of fresh medium and a five channel peristaltic pump were used to feed the five bioreactors at different flowrates.

The continuous experiments were started as batch experiments (paragraph 2.2.5). The feed pump started (beginning of continuous fermentation) only when the fungal growth reached its maximum. Panomalt syrup substrate was used for studying the growth of acidophilic fungus in continuous culture, and the CMC-xylan medium was used for studying enzyme production.

2.3 ANALYTICAL METHODS

2.3.1 pH

The pH of the samples was measured with either a portable pH-meter (Digi-Sense LED pH-Meter) or a Fisher Acumet Model 425 Digital pH/Ion Meter.

2.3.2 Biomass concentration

Biomass concentration in the culture media using soluble substrates was determined by the conventional dry weight method. The samples were filtered through a Gelman Glass Fiber Filter Membrane Type A/E and oven-dried at 105 °C to a constant weight.

2.3.3 Dissolved oxygen concentration, $k_L a$ and Q_{O_2}

The YSI Model 54 Oxygen Meter, the YSI 5400 Series Oxygen/Temperature Probe with the YSI Standard Membrane were used for measuring dissolved oxygen (DO) concentration of the culture medium in the bioreactors. Volumetric oxygen transfer coefficient in liquid culture medium ($k_L a$) and respiration rate or oxygen-uptake rate of the acidophilic fungus ($Q_{O_2} C$) were measured by a dynamic gassing-out method (Atkinson and Mavituna 1983).

2.3.4 ATP concentration

For the determination of ATP concentration, 5 mℓ of the whole culture medium was homogenized on the Model PT 10-35 Polytron Homogenizer (Brinkmann Instruments) using a Model PT 20 ST Saw-Tooth Generator for 30 seconds at medium speed (position 5-6 of speed indicator). One mℓ of this homogenized solution was immediately used for extraction of ATP by following the procedure described in Appendix A.1. The extracted ATP solution was then immediately frozen to -20 °C and then kept at -30 °C for subsequent analysis. When a large quantity of samples were available, they were thawed to 0 °C in an ice bath for ATP measurement using a Model 3000 ATP Photometer (SAI Technology Co.). Detailed procedure for ATP measurement is shown in Appendix A.1.

2.3.5 Protein concentration

Extracellular and free protein concentrations were determined from 1 mℓ of the culture filtrate by following our modified procedure of the modified Lowry method (Lowry et al. 1951; Tan et al. 1984). The culture filtrate samples were kept frozen at -30 °C prior to analysis. When a large quantity of samples were available, they were thawed in a water bath (at room temperature). Detailed procedure for measuring protein concentration are described in Appendix A.2. The crystalline bovine albumin (Sigma) was used to prepare the standard calibration curve.

The precipitation of protein was done using the following method: one mℓ of standard solution containing various concentrations of protein (for the standard calibration curve) or 1 mℓ of culture filtrate (sample) was pipetted into a 1,5 mℓ Eppendorf Micro Test Tube. Following the addition of 0,1 mℓ of 70% trichloroacetic acid (TCA), the tubes were agitated on a vortex mixer for about 10 seconds and then incubated in an ice bath for 60 minutes. The mixtures were then centrifuged at 15 600 x g (15 000 rpm) for three minutes with an Eppendorf Model 5414

Micro Centrifuge. The supernatant in the tubes was then drained and the pellets were rinsed by adding 1 mℓ of cold (0 °C) ethanol (95%)-ether mixture (1:1), then immediately centrifugated again in an Eppendorf Micro Centrifuge for three minutes, and the supernatant was discarded. The tubes were kept at room temperature for 10-15 minutes for complete evaporation of ethanol-ether.

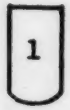
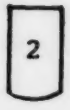
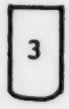
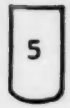
The protein assay was followed by the addition of the following to the tubes: 0,1 mℓ NaOH 1N, then 1,0 mℓ of alkaline copper reagent with the following composition: A (3% Na_2CO_3): B (2% $CuSO_4.5H_2O$): and C (4% Na-tartrate.$2H_2O$) in a 48:1:1 mixture. The tubes were then agitated on a vortex mixer for about 15 seconds and then incubated at room temperature for 10 minutes, then 0,1 mℓ of a 1:3 dilution of a phenol reagent (Folin- Ciocalteau from BDH) was added. The tubes were immediately agitated on a vortex mixer for few seconds and then incubated again at room temperature for 30 minutes. The samples in the tubes were read at 500 nm with a Model Spectronic 710 Bausch & Lomb Spectrophotometer within a total delay of less than 15 minutes.

2.3.6 Enzyme activities

Culture filtrate was used for enzymatic assay for extracellular xylanase, CMCase and FPase. Cellulolytic activities were measured with xylan (Sigma, from larchwood), Na-CMC (Sigma, low viscosity) and Whatman No. 1 filter paper as substrates, respectively. The detailed procedures for cellulolytic enzyme assays are shown in Table 2.2 and in Appendix A.3. These were modified procedures adopted from those described by Khan et al. 1981 and Mandels et al. 1976. Enzyme activity was calculated using the formula shown in Table 2.2. One international unit (IU) of enzyme activity was expressed as 1 µmol of glucose equivalent of reducing sugar released per mℓ of enzyme sample per minute of reaction time, under assay conditions. The reducing sugar was measured with dinitrosalicylic acid (DNS) reagent (Miller 1959) and color was read at 575 nm on a Bausch & Lomb Spectronic 710 Spectrophotometer.

Table 2.2 - Cellulolytic enzyme assay methods.

Step 1: For each sample of medium culture filtrate (containing cellulolytic enzymes E to be assayed), the following tubes are prepared:

Tube No.:	 1	 2	 3	4	 5
Used for:	Zeroing spectrophotometer	Test sample	Enzyme control	Substrate control	Standard calibration curve
Symbol:	Z	T	CE	CS	SC
Content:					
1,0 mL	Glucose buffer[a]	Glucose buffer[a]	Glucose buffer[a]	Glucose buffer[a]	Glucose buffer[a]
0,5 mL	Buffer[b]	Substrate[c]	Buffer[b]	Substrate[c]	Buffer[b]
0,5 mL	Distilled water	Culture medium filtrate[d]	Culture medium filtrate[d]	Distilled water	Glucose standard solution[a]

Step 2: Incubation at T°C for t min.[c]

Step 3: Stop the enzyme reaction and develop the color reaction for detecting the reducing sugars, by

(a) adding 2 mL of DNS reagent to each tube,
(b) then incubating the tubes at 100°C for 15 min,
(c) then cooling the tubes to room temperature with cold water.

Table 2.2 - Cellulolytic enzyme assay methods (continued).

Step 4: Read the absorbance at 575 nm

Tube No.:	1	2	3	4	5
	Adjust Z = 0,000	Read T	Read CE[f]	Read CS[g]	Read SC[e]

Step 5: Calculations.

The net quantity of reducing sugars produced by the enzymatic (cellulolytic) reactions during the enzyme assay is

$$Q \text{ (absorbance)} = T - CE - CS \qquad (1)$$

The enzyme activity is

$$IU \left(\begin{array}{l}\mu\text{mol reducing sugars} \\ \text{released/mL-min}\end{array}\right) = \frac{\begin{array}{c}\text{reducing sugar concentration} \\ \text{corresponding to Q (g/L)}\end{array} \times 1\,000}{\text{incubation time (min)} \times 180} \qquad (2)$$

Legends for Table 2.2 :

(a) For compensating the glucose loss by oxidation or by adsorption on DNS reagent. For the FPase activity measurement, this quantity of glucose buffer was added to all tubes between the steps (3a) and (3b).

(b) Acetate buffer at pH 3,0 (optimum pH of the acidophilic fungus enzymes).

(c) Substrate and incubation conditions:

Activity	Substrate	Temperature	Time
CMCase	Na-CMC (1% in buffer)	50°C	15 min
Xylanase	Xylan (1% in buffer)	50°C	15 min
FPase	Whatman No 1 filter paper (1 cm × 6 cm)	50°C	60 min

(d) Filtration through Type A/E Gelman fiber glass membrane filter.

(e) For spot-checking the standard calibration curve of glucose.

(f) The enzyme control CE measures the reducing sugar produced in the absence of substrate during the incubation period for enzyme assay. This is due to the enzymatic reactions between the enzymes (in the culture filtrate) and the residual cellulosic / lignocellulosic substrate initially present in the culture filtrate.

(g) The substrate control CS measures the reducing sugar produced by partial physico-chemical hydrolysis of substrate under assay conditions (e.g. high temperature, very low pH), in the absence of enzymes. It also measures the reducing sugar initially associated with substrate as impurity. The CS value also serves as a correction factor for compensating any interference between substrate and DNS reagent, if it occurs.

For very low levels of enzyme activity, an arbitrary but convenient unit of enzyme activity U = 180 x IU was used. Thus, one unit (U) of enzyme activity was expressed as 1 µg of glucose equivalent of reducing sugar released per mℓ of enzyme sample per minute of reaction time.

2.3.7 Contamination

A streak-plate method was used to observe the contamination level of the culture medium sample. A loopful of whole culture medium was uniformly smeared on the surface of a MEA-agar (2%) medium. The petri dishes were then incubated at 30 °C and observed daily.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 ON THE ANALYTICAL METHODS

3.1.1 Protein concentration

The development of a suitable method for the determination of a very low level (<50 mg/ℓ) of protein concentration in the culture filtrate is essential for this project. Furthermore, a more convenient method which requires no transfer of samples between containers (test tubes, centrifugation tubes, etc.) is prefered to avoid sample-loss due to such transfers. This way the handling of glassware will also be minimized. The modified method for the determination of protein concentration as described in paragraph 2.3.6 and in Appendix A.2 has been based on the above objective.

Because the culture medium of acidophilic fungus was acidic, the first tests were conducted to find out whether or not this acidic pH interferes with calibration curve. It was found that the standard calibration curve using bovine albumine at pH 2,0 is identical to that using bovine albumine at its natural pH. Thus, the acidic pH did not interfere with protein measurement.

With respect to the modified Lowry method (Tan et al. 1984), two following major modifications have been made:

(1) the protein precipitation time with TCA of 60 minutes was selected instead of 15 minutes. As shown in Figure 3.1 and Table B.1,

the precipitation time of 15 minutes was not long enough to detect full protein content in the solution;

(ii) the TCA precipitated protein was rinsed with cold ethanol-ether instead of cold 7% TCA solution. The advantage of this modification is that ethanol-ether could be easily and completely removed from a protein sample by simple natural evaporation at room temperature.

With respect to the existing chemical manual methods for protein determination, the most significant improvement of our developed method lies in the fact that all steps of the analytical procedure were done in one 1,5 mℓ Eppendorf Micro Test Tube. This improved procedure has the advantage of no sample (protein) loss due to transfers and less handling of glassware.

3.1.2 DO concentration, $k_L a$ and Q_{O_2}

The DO concentration-monitoring with the YSI DO probe during the fermentation was very satisfactory, providing that the membrane was replaced once a day.

The dynamic gassing-out method for estimating $k_L a$ and Q_{O_2} was quite convenient owing to its simple procedure and its not requiring of sophisticated analytical instruments. However, this method is severely limited and cannot be used in fermentation broth containing high concentration of organic substances, where the (saturated) DO level is less than 1 mg/ℓ. Such is the case when acidophilic fungus is grown in glucose medium or in Panomalt syrup medium.

The typical DO profile curves obtained by the dynamic gassing-out method for the CMC-xylan and newspaper media are shown in Figures 3.2 and 3.3 respectively. On these curves, the overdrop portion of approximately 3 mg/ℓ immediately following the air cut-off and the overshot portion of approximately 3,5 mg/ℓ immediately following the reaeration

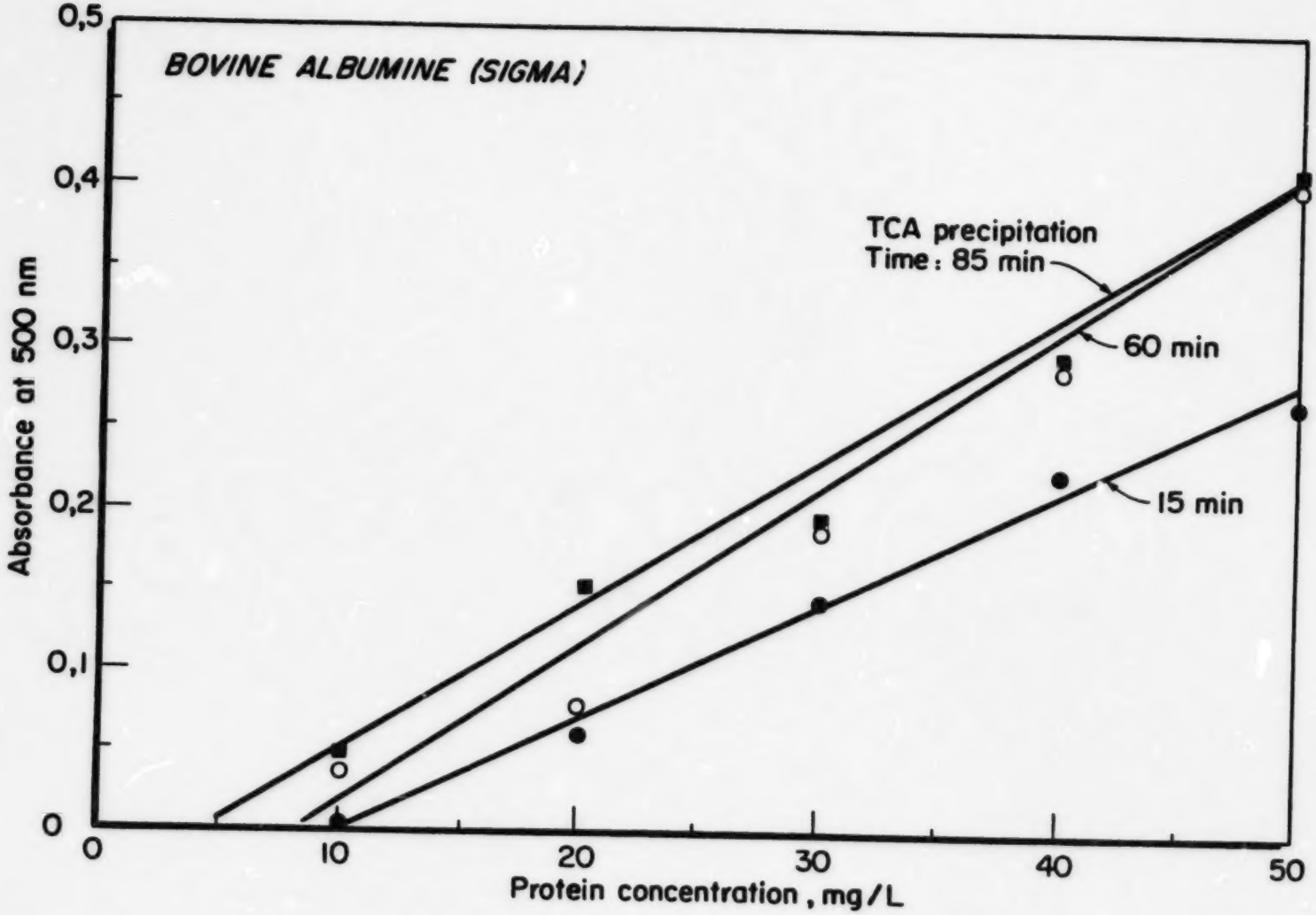


Fig. 3.1 - Effect of TCA precipitation time on the standard calibration curve for protein.

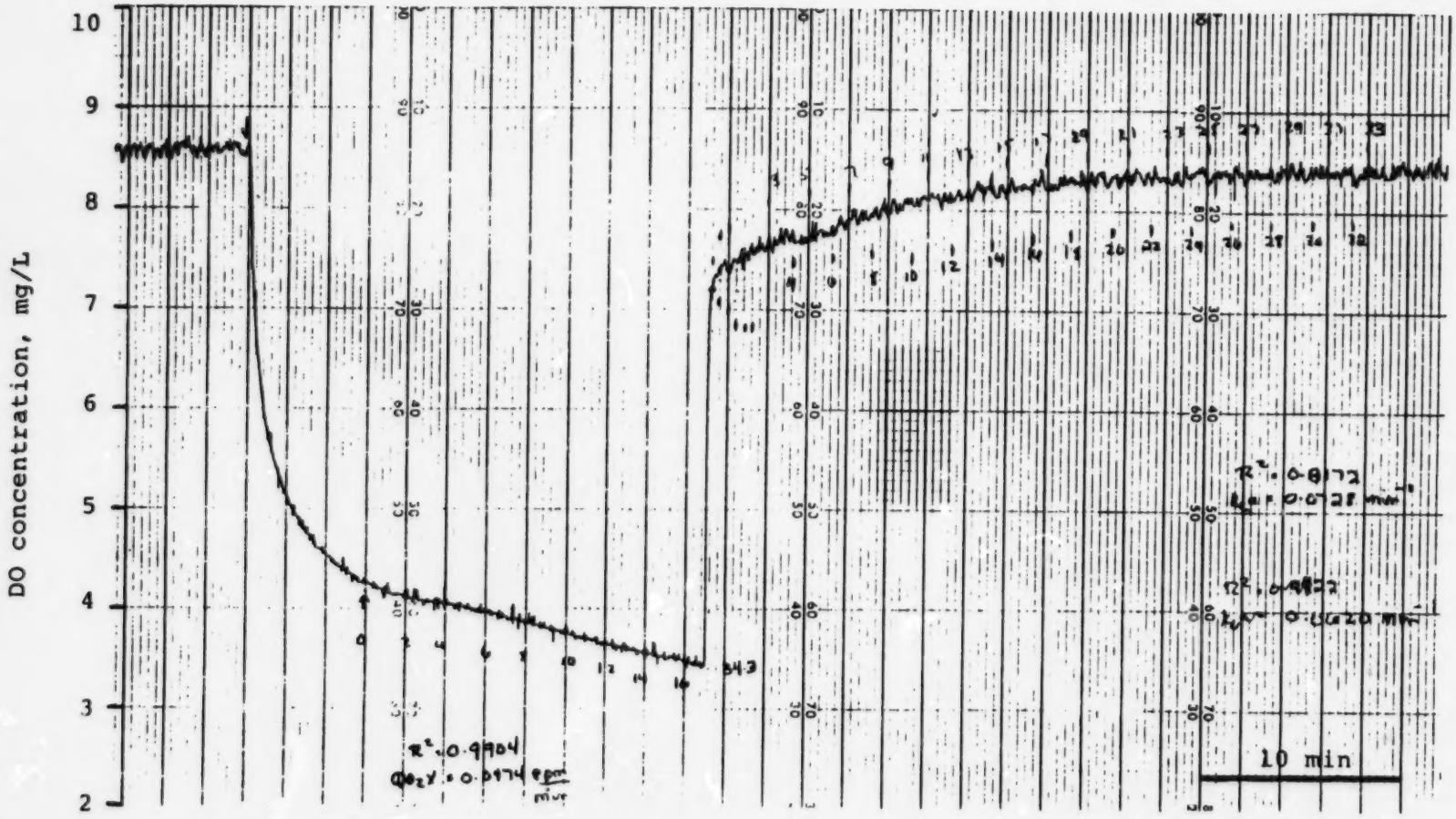


Fig. 3.2 - Typical dynamic gassing-out curve for DO concentration in CMC-xylan medium : Bioreactor 2A at 211 h.

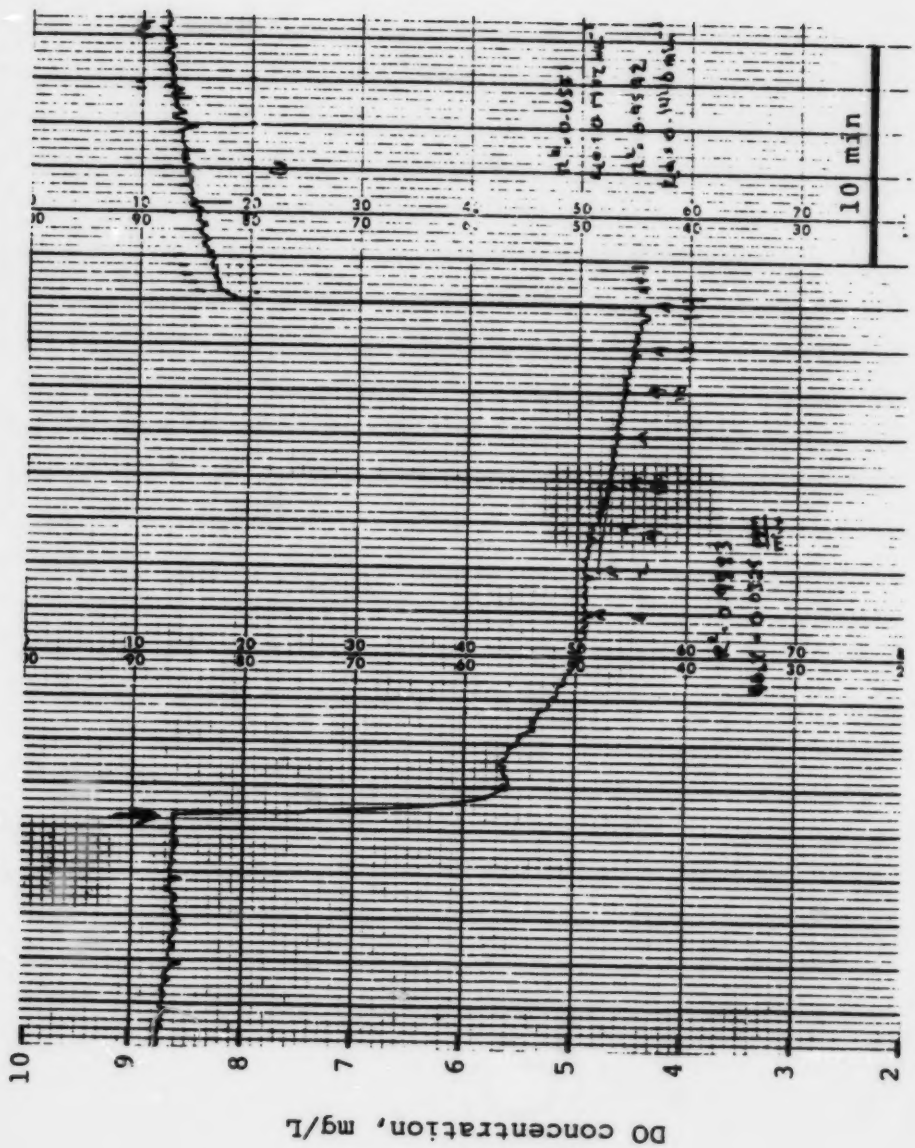


Fig. 3.3 - Typical dynamic gassing-out curve for DO concentration in newspaper medium : Bioreactor 1B at 214 h.

were not used for the calculation of $k_L a$ and $Q_{O_2} C$. Such overdrop and overshot portions of the curves represented a level of oversaturation of oxygen in the culture medium under fermentation conditions at the time of measurement. The oversaturation of oxygen detected by the DO probe was caused by the presence of micro- and/or mini-air bubbles in the liquid medium during fermentation.

3.1.3 Enzyme activities

3.1.3.1 Precision of the FPase assay

The method of FPase assay developed by Mandels et al. 1976 was selected for this work because of its popularity in the scientific community. Tests were conducted in the first place to evaluate the precision of the FPase assay method as manually performed by our research staff in the laboratory.

The results in Table 3.1 show that higher precision on the FPase assay was obtained with samples containing a higher concentration of enzymes. Indeed, a very good coefficient of variation (3,3%) was obtained in samples containing 1,1 IU, while a very poor coefficient of variation (50,9%) was obtained with samples containing only 0,01 IU. The same order of standard deviations obtained with these samples indicated that the precision of the method could not be improved for the measurement of very low concentrations of enzyme.

3.1.3.2 Effect of dilution on the FPase assay

The nonlinear effects of dilution (or enzyme concentration) on the cellulolytic enzyme assays (xylanase, CMCase and FPase) are illustrated in Figure 3.4 (and shown in Table B.2). This phenomenon has been reported for xylanase and CMCase (Cauchon and LeDuy 1984). The results in Figure 3.4 indicate that when samples contain either too much or too little enzyme diluted or concentrated prior to the assay procedure, a

Table 3.1 - Precision of the FPase assay method[a].

	Mean from 10 tubes $\bar{x}$	Standard deviation s	Coefficient of variation (%) $\frac{s}{\bar{x}}$
Enzyme sample A[b]:			
Weight of filter paper strip (g)	0,055	0,001	1,9
Absorbance at 575 nm:			
CS	0,141	0,051	36,2
CE	0,703	0,026	3,7
T	0,856	0,034	4,0
Activity (U/mL)	1,137	0,579	50,9
(IU/mL)	0,01		
Enzyme sample B[c]:			
Weight of filter paper strip (g)	0,056	0,003	4,4
Absorbance at 575 nm			
CS	0,053	0,018	38,4
CE	0,017	0,010	59,6
T	1,219	0,040	3,3
Activity (U/mL)	20,56	0,674	3,3
(IU/mL)	0,1		

(a) Details of the assay method are in Table 2.2.

(b) Culture filtrate of acidophilic fungus grown in starch medium at pH 2,0 for 14 days.

(c) Commercial *Trichoderma reesei* cellulase (Biocon) solution 0,02% w/v.

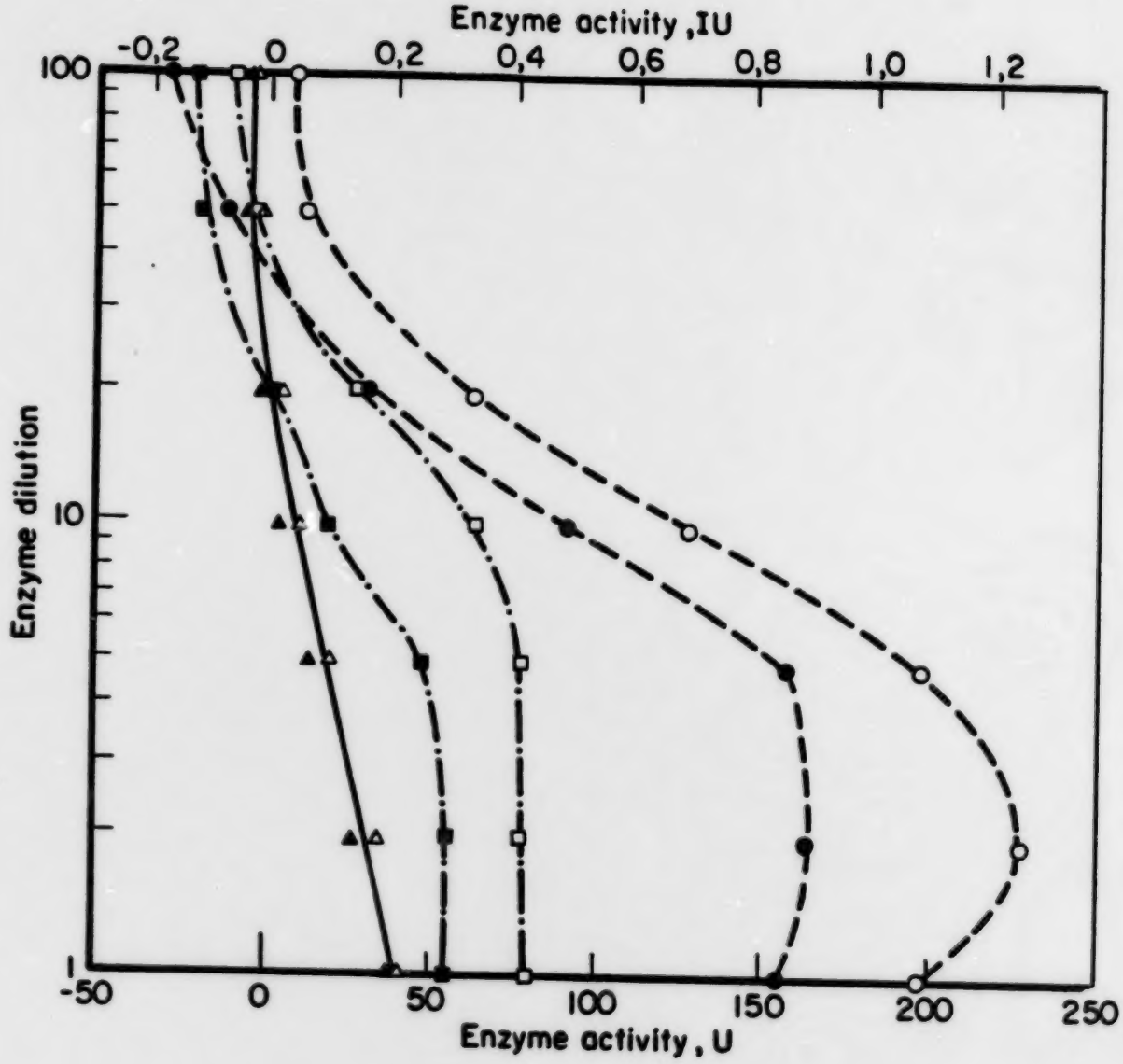


Fig. 3.4 - Effect of enzyme dilution on the measured enzyme activities.
Legends: Xylanase (o, ●); CMCase (□, ■); FPase (△, ▲);
Run 1 (o, □, △); Run 2 (●, ■, ▲).
More details are in Table B.2.

conventional multiplicative correction factor (for diluted samples) or divisional correction factor (for concentrated samples), successfully used for other enzyme assays, cannot be applied for cellulolytic enzymes.

3.1.3.3 Negative values of enzyme activity

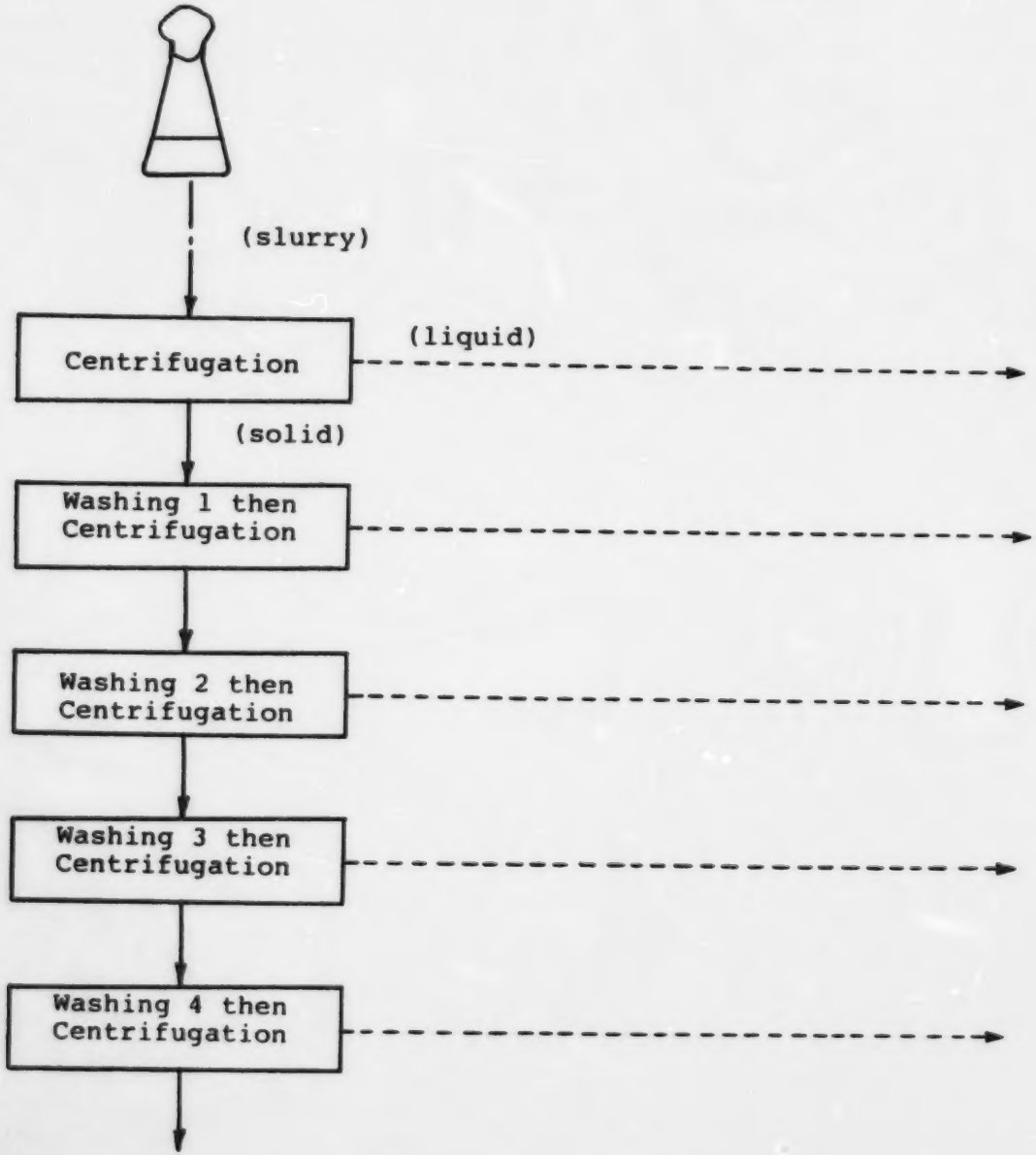
The method of cellulolytic enzyme assays shown in Table 2.2 took into account two correction factors: a control for substrate CS and a control for enzyme CE. Most of the time during this project, the assay results on the culture filtrates of the acidophilic fungus showed very significant values of the CS and CE readings, with CS > 2CE in general. This double correction resulted in negative values of cellulolytic activities when the enzyme concentration in the sample was very low (samples at dilutions 50 and 100 in Figure 3.4). This is because the reading value T of the test sample was less than the sum of two correction readings CS and CE [see formula (1) in Table 2.2]. This problem had not been previously reported because (i) only one correction factor, either CS or CE was used by other workers; and (ii) enzyme activity was relatively high (therefore high values of the reading T) in their samples.

3.1.3.4 Miscellaneous tests on enzyme assays

A series of experiments designed for testing several points related to the cellulolytic enzyme assays is shown in Figure 3.5. The results on CMCase and xylanase are also shown in the Figure 3.5.

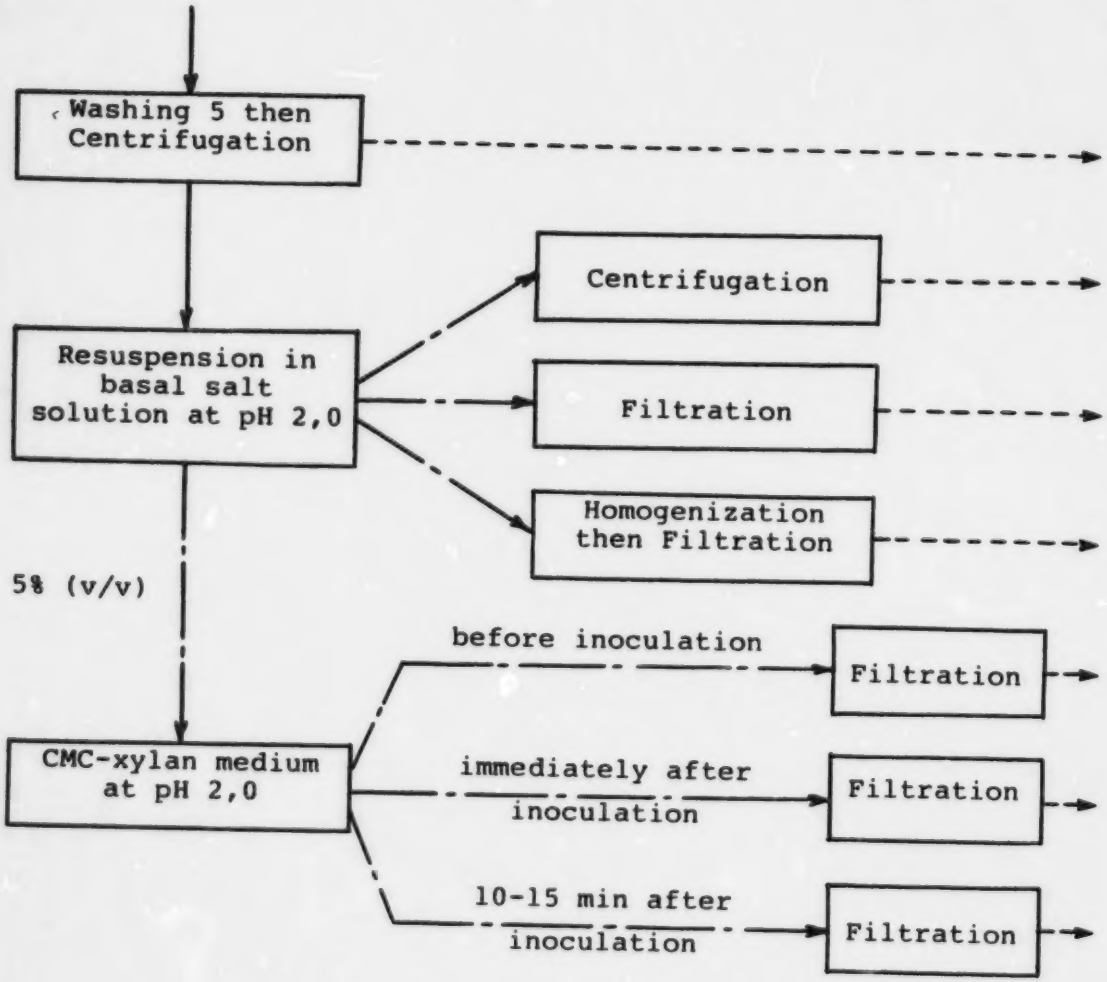
The effect of the washing of inoculum prior to inoculating the culture medium in order to remove cellulolytic enzymes which were absorbed by the inoculating cells was observed by comparing the enzyme activities of samples A to F. It was found that two washings are essential for removing the xylanase adhering to inoculating cells. The

Fig. 3.5 - Miscellaneous tests on enzyme assays.



Sample	Enzyme activity (U)	
	CMCase	Xylanase
A	1,7	25,8
B	0,7	8,9
C	3,2	3,0
D	1,7	2,1
E	2,6	2,1

Fig. 3.5 - Miscellaneous tests on enzyme assays (continued).



F	3,2	2,6
G	2,3	2,2
H	0,9	3,8
L	4,5	14,8
I	-1,5	3,9
J	3,5	3,8
K	3,4	3,5

(*) All washings were done with one volume of basal salt solution at pH 2,0.

results were not conclusive CMCase because of the very low level of this enzyme found with the inoculating cells.

Comparison of the results of samples G and H revealed that filtration and centrifugation were equivalent for separating cells from culture medium. The culture filtrate did not contain significantly less amounts of enzyme than the culture supernatant. This suggested that the glass-fiber filter membrane used for solid-liquid separation in our work did not significantly absorb and/or absorb cellulolytic enzymes of acidophilic fungus, under such analytical conditions.

The results of samples H and L showed that the acidophilic fungus contained some amounts of intracellular and/or cell membrane associated CMCase and xylanase.

The results of samples H, I J and K indicated that CMC-xylan mixture did not have induction power over the acidophilic fungus.

3.1.4 Screening tests for mutant selection

A series of studies have been conducted to establish a rapid and convenient semi-quantitative method for screening the positive mutants of acidophilic fungus. A positive mutant was defined as that which produces a large amount of extracellular FPase activity in a culture medium at pH 2,0.

The dye used for testing and developing a screening test was the Remazol Brilliant Blue R, and the procedure for the preparation of the dyed cellulose (cellulose azure) was the one described by Poincelot and Day 1972. Congo Red, which is commonly used for cellulolytic enzymes from other microorganisms (Teather and Wood 1982), cannot be used for our study because it is precipitated at pH 2,0, which is our selection condition.

The following methods have been tested:

(i) Screening test on petri dishes using undyed filter paper fibers, and both dyed and undyed α-cellulose as the sole carbon source in solid agar medium at pH 2,0. The preparation of filter paper fibers was the one developed by Daigneault-Sylvestre and Kluepfel 1979. The principle of the method is shown in Figure 3.6 A. The detection of a positive mutant was based on the clear zone (for undyed substrate) or on the colored zone (for dyed substrate) associated with the colony.

(ii) Screening test on petri dishes using dyed filter paper. This method is a modification of the above method (i), in which the whole dyed filter paper was used instead of just its fibers. The dyed filter paper was laid either on the top or at the bottom of the agar layer containing basal salts at pH 2,0. The principle of the method is shown in Figure 3.6 B and C. The detection of a positive mutant was based on the colored zone above (dyed filter paper at the bottom) or below (dyed filter paper on the top) the colony.

(iii) Screening test on solid medium in test tubes using dyed α-cellulose according to the method established by Smith 1977. The principle of the method is illustrated in Figure 3.6 D. The detection of a positive mutant was based on the colored zone underneath the colony.

(iv) Screening test in liquid medium in test tubes using a strip of dyed filter paper as substrate according to the method described by Poincelot and Day 1972. The principle of the method is illustrated in Figure 3.6 E. The detection of a positive mutant was based on the intensity of the released color in the liquid medium.

It was found that none of the tests on solid medium (agar) were applicable because of the very slow growth rate of the acidophilic fungus and also because of its very low level of extracellular FPase.

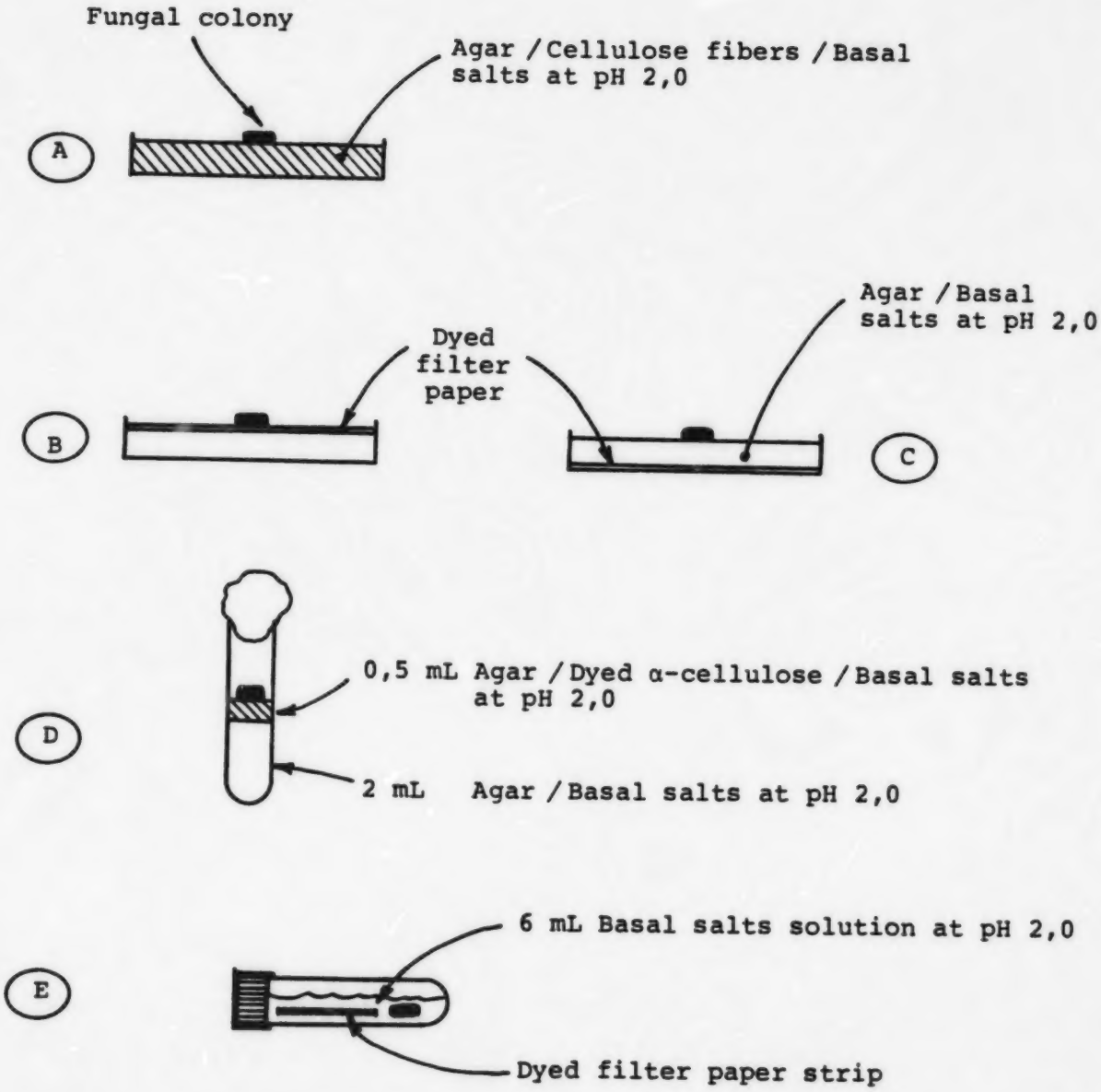

Fungal colony
Agar / Cellulose fibers / Basal salts at pH 2,0
A
Agar / Basal salts at pH 2,0
Dyed filter paper
B
C
0,5 mL Agar / Dyed α-cellulose / Basal salts at pH 2,0
D
2 mL Agar / Basal salts at pH 2,0
6 mL Basal salts solution at pH 2,0
E
Dyed filter paper strip

Fig. 3.6 - Methods for screening of positive mutants.

The clear zones or the decolored zones were not easily recognizable after a long incubation period up to two weeks.

Some weak releases of the dye color were observed with the screening tubes containing liquid medium (method iv). However, this weak color obtained might be the result of the very low level of acidophilic fungus FPase or the low in solubility (precipitation) of Remazol Brilliant Blue R in an acid medium at pH 2,0 or both.

An experiment was conducted to find out the effects of basal salts present in the culture medium and extreme pH (very acidic and very basic) on the precipitation of Remazol Brilliant Blue R dye. A very insignificant amount of precipitation was observed in a dye solution prepared the conventional way and stored at room temperature for three days. The same phenomenon was observed with the dye solution adjusted to pH 1,0 prior to storage. Therefore, it was concluded that the acidic pH of a solution and the sulfuric acid used to adjust pH did not precipitate Remazol Brilliant Blue R dye. When the dye solution contained basal salts equivalent to those present in the culture medium, abundant quantities of precipitate were observed in these solutions (at pH 1 and at pH 11) after three days of storage at room temperature. Thus, under very acidic conditions Remazol Brilliant Blue R was precipitated by the basal salts in the culture medium. It is therefore not suitable for use in the dye-released method for screening the positive mutants of acidophilic fungus.

The dye release method was, however, used simultaneously with the conventional FPase assay (Mandels et al. 1976) for screening the positive mutants during mutation tests in order to find the correlation between released color and level of FPase activity of the acidophilic fungus. The results are discussed in paragraph 3.2.3.

3.2 ON THE IMPROVEMENT OF ENZYME PRODUCTION BY INDUCTION AND BY MUTATION

3.2.1 Enzyme production on various substrates

No extracellular cellulolytic enzymes were produced with any of the monosaccharides, disaccharides and starches tested. Very low concentration of residual sugar in the culture filtrate on the 14th day (approximately 1 g/ℓ in all media, from an initial substrate concentration of 10 g/ℓ) indicated that most of these carbon sources had been used for cell growth.

None of the cellulosic and lignocellulosic substrates tested (Figure 3.7; Tables B.3 to B.5) significantly produced FPase activity (less that 0,025 IU). The level of CMCase activity in the culture filtrate was also very low. Xylan, newsprint and CMC-xylan mixture produced approximately 0,05 to 0,1 IU of CMCase activity (Figure 3.7). The media containing xylan (xylan alone and CMC-xylan mixture) gave the highest xylanase activity (0,5 to 0,8 IU) on the 7th day of fermentation. Other cellulosic and lignocellulosic substrates produced lower levels of xylanase activity (less than 0,2 IU).

All of the above observations correspond very well with those previously reported for acidophilic fungus (Cauchon and LeDuy 1985). The following conclusions could be drawn: (1) xylan has to be used as part of the substrate for the production of high xylanase activity broth; and (ii) no evidence on any trend or correlation among the three enzyme activities (xylanase, CMCase, FPase) is observed.

Based on the above results, two selected substrates for studying the fermentation kinetics in the bioreactors are: CMC-xylan mixture as model for simple cellulosic substrates; and newspaper as model for complex lignocellulosic substrates.

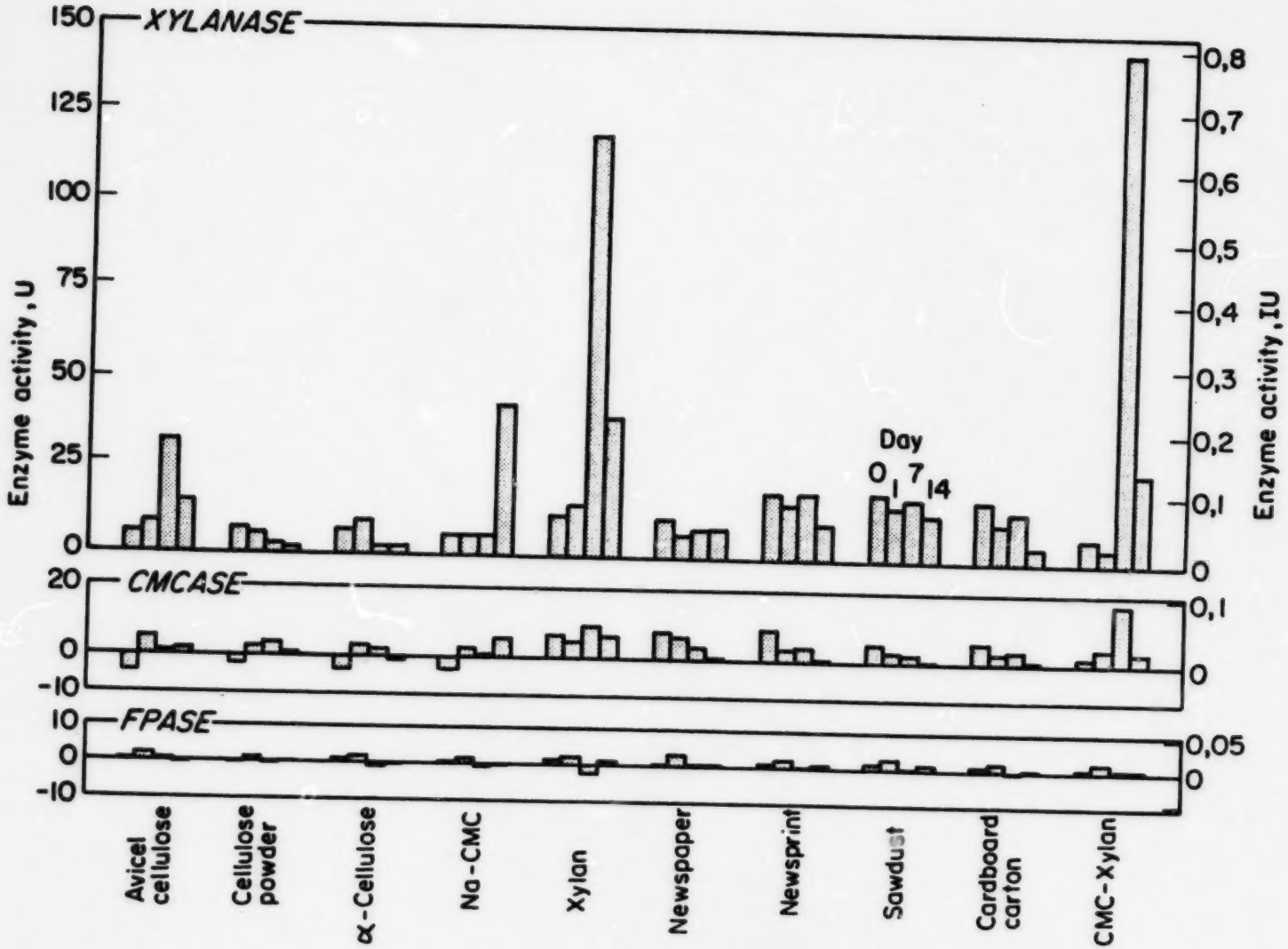


Fig. 3.7 - Enzyme production from various substrates.

3.2.2 Induction

Results of the induction tests with cellobiose, Na-CMC, xylan, newspaper, and sawdust (Figures 3.8 to 3.12; Tables B.6 to B10) revealed that none of these has inducing power over the synthesis and excretion of cellulolytic enzymes (xylanase, CMCase, FPase) of acidophilic fungus. The results are consistent with those of the tests conducted on the production of enzymes on various substrates (Figure 3.7), where no significant change in the level of enzyme activities occurred between day 0 and day 1.

3.2.3 Mutation

The number of survival colonies obtained from the mutation tests with various physical and chemical methods are shown in Tables 3.2 to 3.6. Attempts to produce the conventional log-survival versus treatment dose curves were unsuccessful. However, all survival colonies resulted from the mutation tests had less than one percent of survival chance, as indicated by the results in Tables 3.2 to 3.6.

FPase activity of all survival colonies from the mutation tests was very low (less than 0,01 IU). This level of FPase activity was in the same order as that of untreated colonies (colonies at time zero) as shown in Figures 3.13 to 3.17. It was concluded that a mutant of acidophilic fungus NC-11 with high extracellular FPase activity (our objective) could not be obtained.

The plots of FPase activity versus the released color of dyed filter paper (Figures 3.13 to 3.17) suggested that there is no correlation between them. While the dyed-released method has been successfully used in screening of cellulolytic enzymes from other microorganisms(Leisola and Lingo 1976; Leisola and Linko 1977; Smith 1977; Daigneault-Sylvestre and Kluepfel 1979; Ng and Zeikus 1980), it does not work with acidophilic fungus. This is because of the fungus very

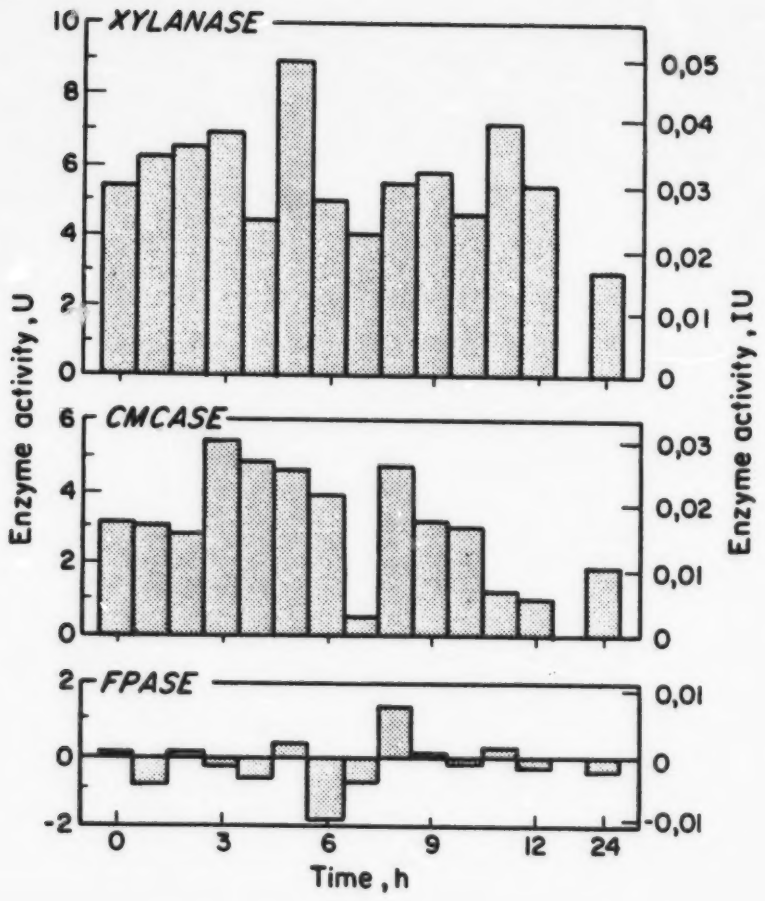


Fig. 3.8 - Induction test with cellobiose.

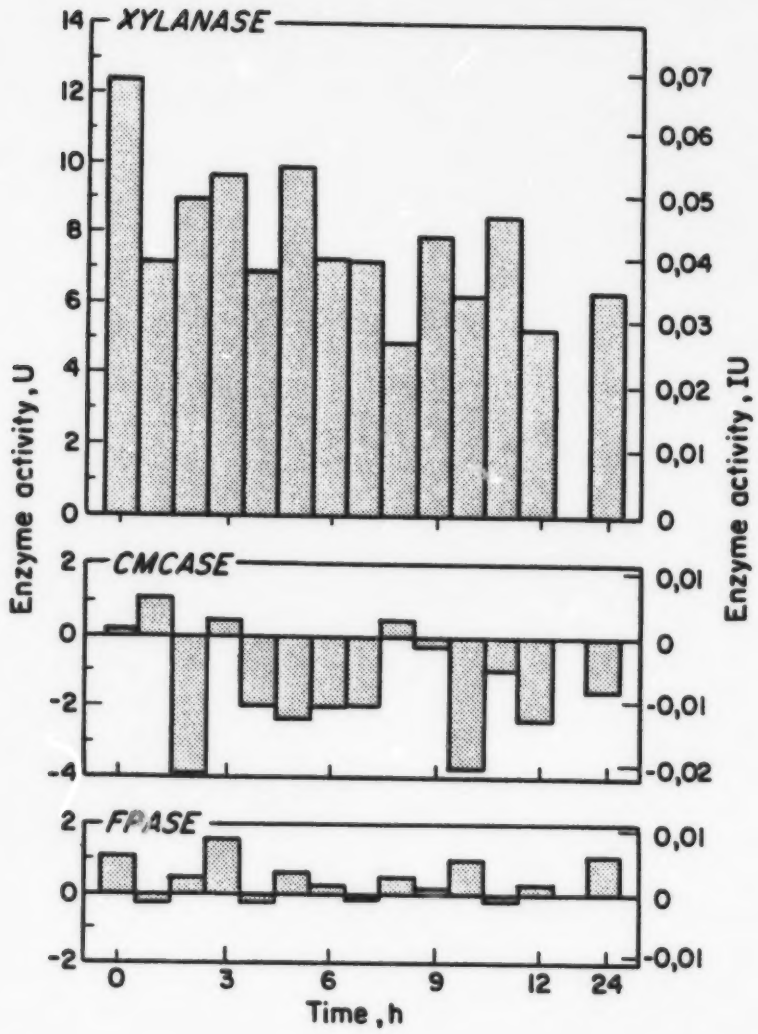


Fig. 3.9 - Induction test with Na-CMC.

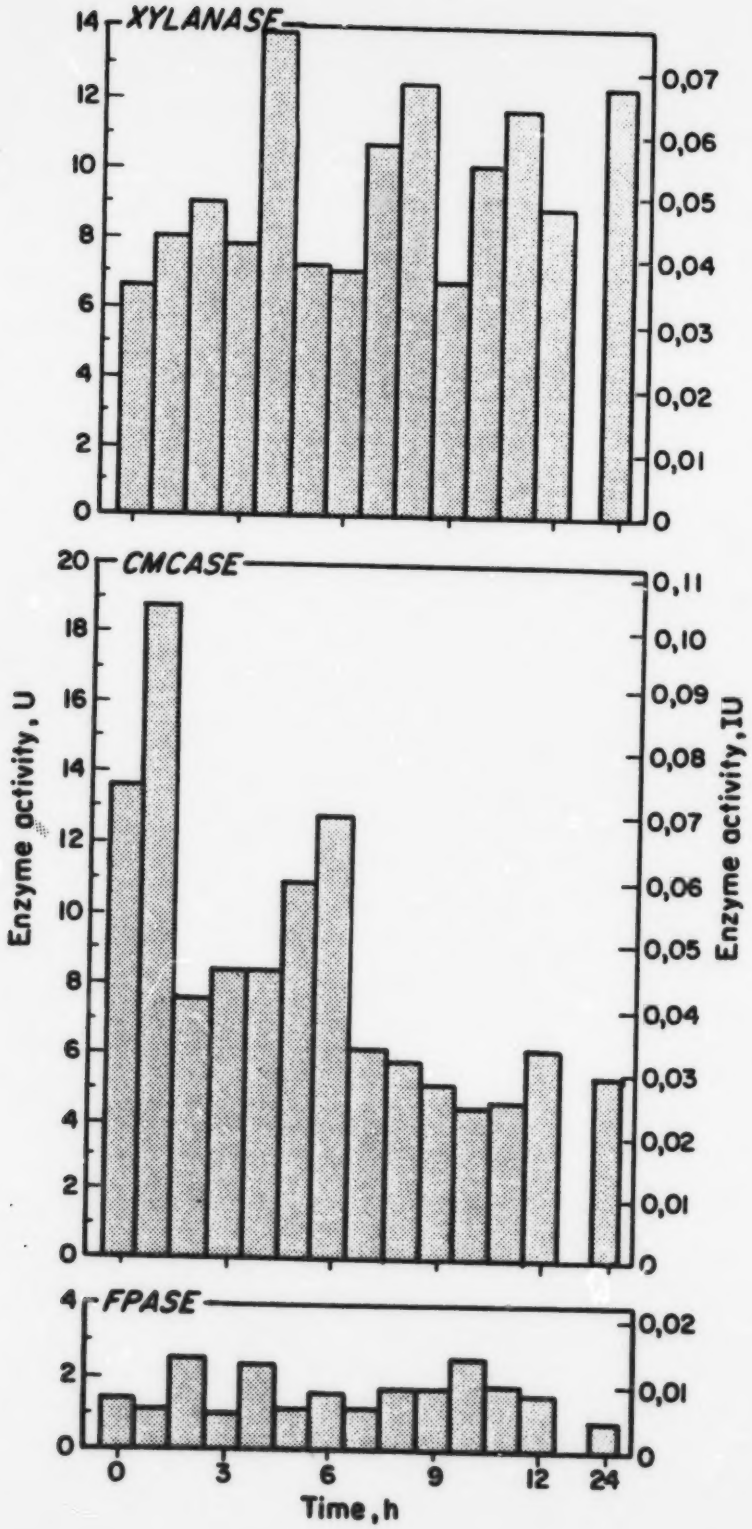


Fig. 3.10 - Induction test with xylan.

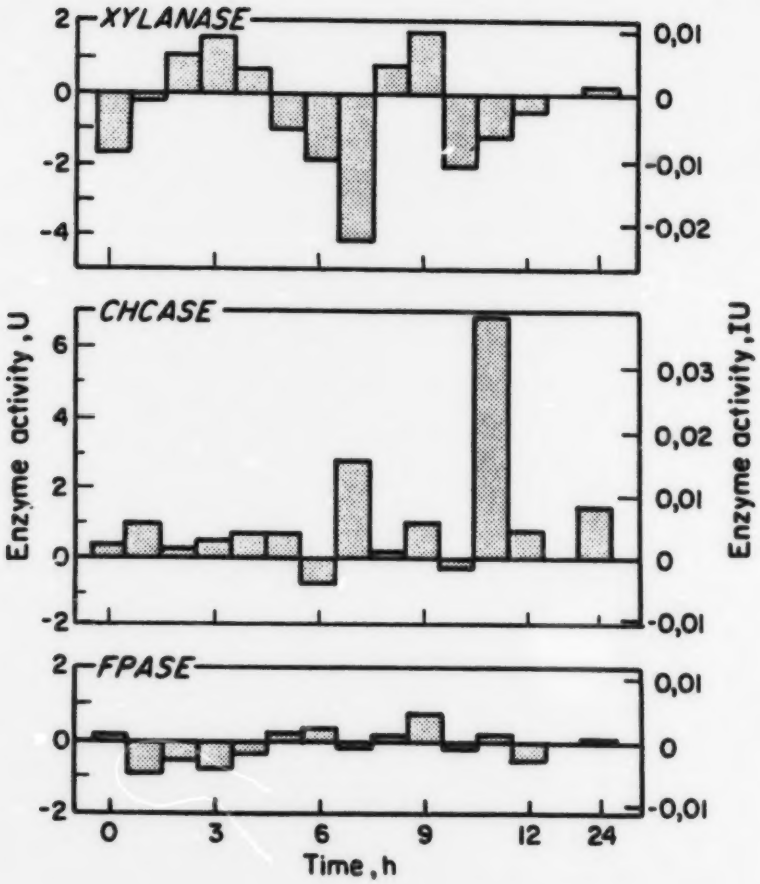


Fig. 3.11 - Induction test with newspaper.

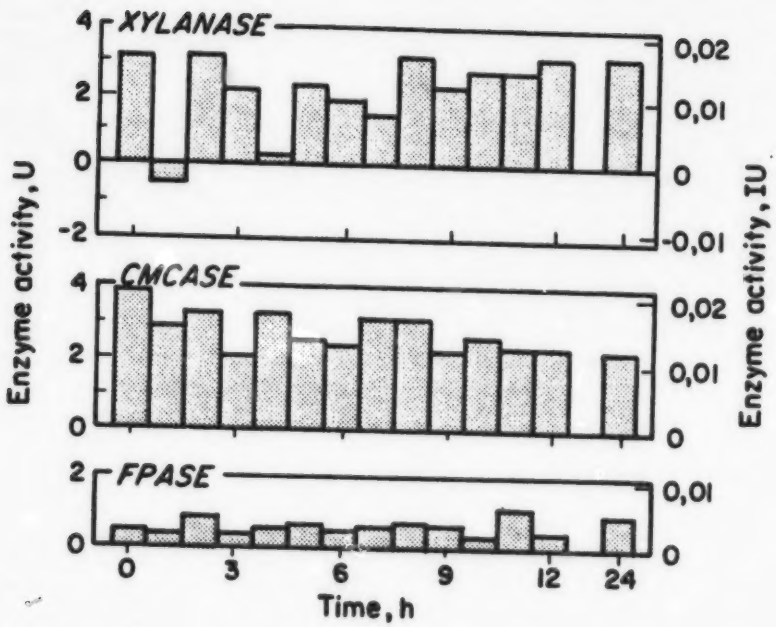


Fig. 3.12 - Induction test with sawdust.

Table 3.2 - Mutation tests with UV : Number of survival colonies.

Exposure time (h)	Petri dish No 1	No 2	No 3	No 4	No 5
0	overgrown	overgrown	overgrown[a]		
0,5	0	1[a]	0	0	0
1	1	1	4	3	2
2	0	1	0	0	0
3	0	0	0	0	0
4	0	0	0	0	0

(a) Slightly contaminated (*Aspergillus* ssp.).

Table 3.3 - Mutation tests with X-ray : Number of survival colonies.

Exposure time (min)	No 1	No 2	No 3	No 4	No 5	No 6
FIRST TEST:						
0	overgrown	overgrown	overgrown	overgrown	overgrown	overgrown
2	2	5	4	2	11	5
10	1	0	0	0	0	0
30	1	0	0	0	0	0
60	1	1	2	0	0	0
135	0	0	0	0	0	0
SECOND TEST:						
0	overgrown	overgrown	overgrown	overgrown	overgrown	overgrown
2	2	0	0	0	1	1
10	0	4	0	0	0	0
30	1	1	0	0	0	0
60	6	1	1	1	0	0

Table 3.4 - Mutation tests with EMS : Number of survival colonies.

Exposure time (h)	First test					Second test				
	Petri dish					Petri dish				
	No 1	No 2	No 3	No 4	No 5	No 1	No 2	No 3	No 4	No 5
0	overgrown	overgrown	overgrown	overgrown	overgrown	overgrown	overgrown	overgrown	overgrown	overgrown
0,5	85	80	90	100	100	214	134	153	162	150
1	a	a	a	a	a	63	47	47	48	38
2	3	4	1	8	2	16	10	19	13	11
3	1	0	0	0	0	5	0	1	2	2
4	2	1	0	0	0	2	0	0	0	0

(a) Contaminant overgrown (*Aspergillus* spp.).

Table 3.5 - Mutation tests with NTG : Number of survival colonies.

Exposure time (min)	First test					Second test				
	Petri dish					Petri dish				
	No 1	No 2	No 3	No 4	No 5	No 1	No 2	No 3	No 4	No 5
0	overgrown	overgrown	overgrown	overgrown	overgrown	overgrown	overgrown	overgrown	overgrown	overgrown
15	0	1	0	1	0	0	0	0	0	0
30	0	0	0	1	1	1	0	0	0	0
45	0	0	0	0	0	1	0	0	0	0
60	2	1	0	2	1	1	0	0	0	0
75	2	0	0	0	1	0	0	0	0	0

Table 3.6 - Mutation tests with nitrous acid : Number of survival colonies.

Exposure time (h)	Petri dish No 1	No 2	No 3	No 4	No 5
FIRST TEST:					
0	overgrown	overgrown	overgrown	overgrown	overgrown[a]
0,5	0	0	0	0	0
1	0	0	0	1	1
2	2	0	0	0	0
3	0	0	0	0	0
4	0	0	0	0	0
SECOND TEST:					
0	overgrown	overgrown	overgrown	overgrown	overgrown
15	2	0	0	0	0
30	0	0	0	0	0
45	1	0	0	0	0
60	0	0	0	0	0
75	0	0	0	0	0

(a) Slightly contaminated (*Aspergillus* spp.).

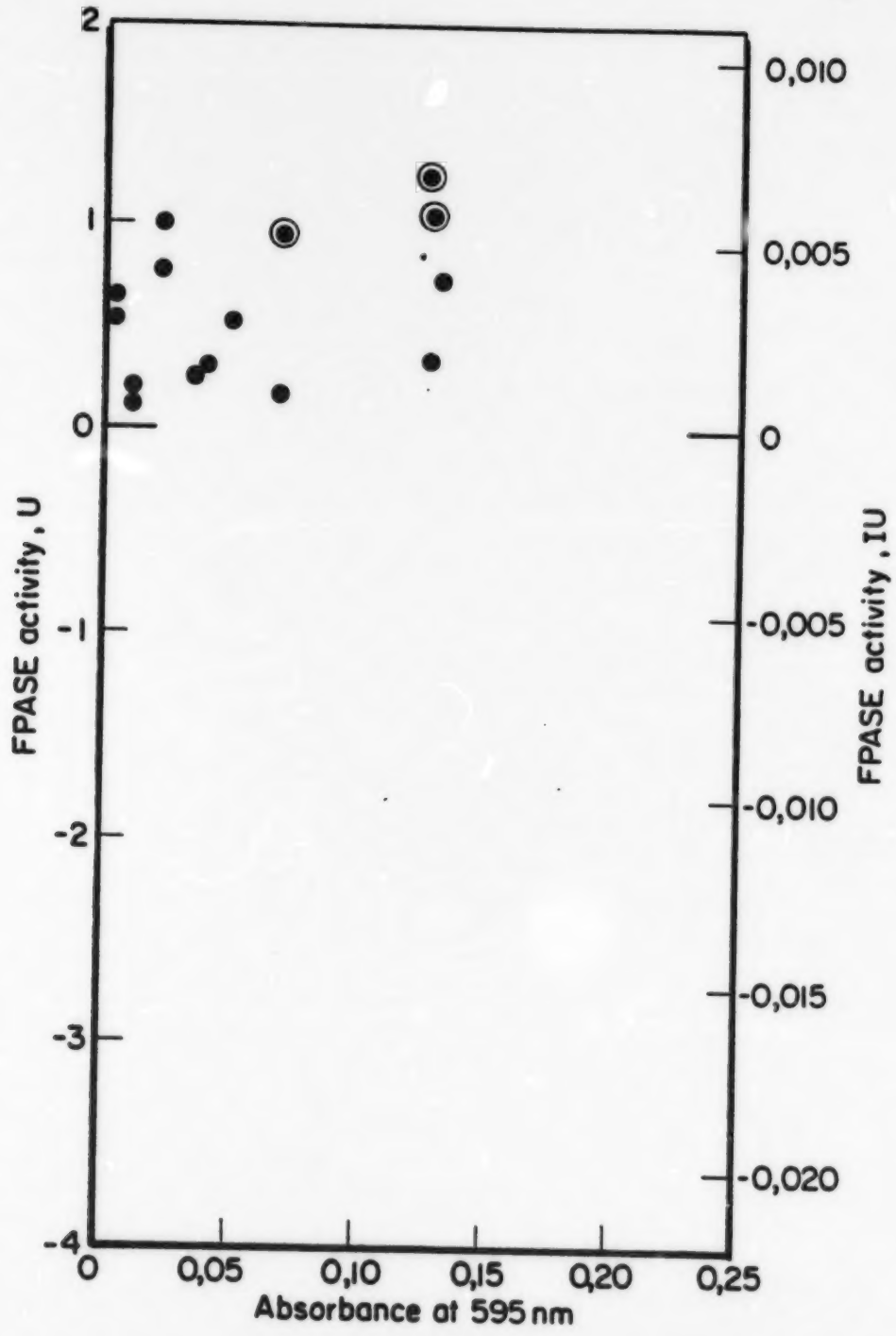


Fig. 3.13 - Mutation tests with UV : Relation between FPase activity and released color of survival colonies.
Legends: Colonies at time zero (◉).

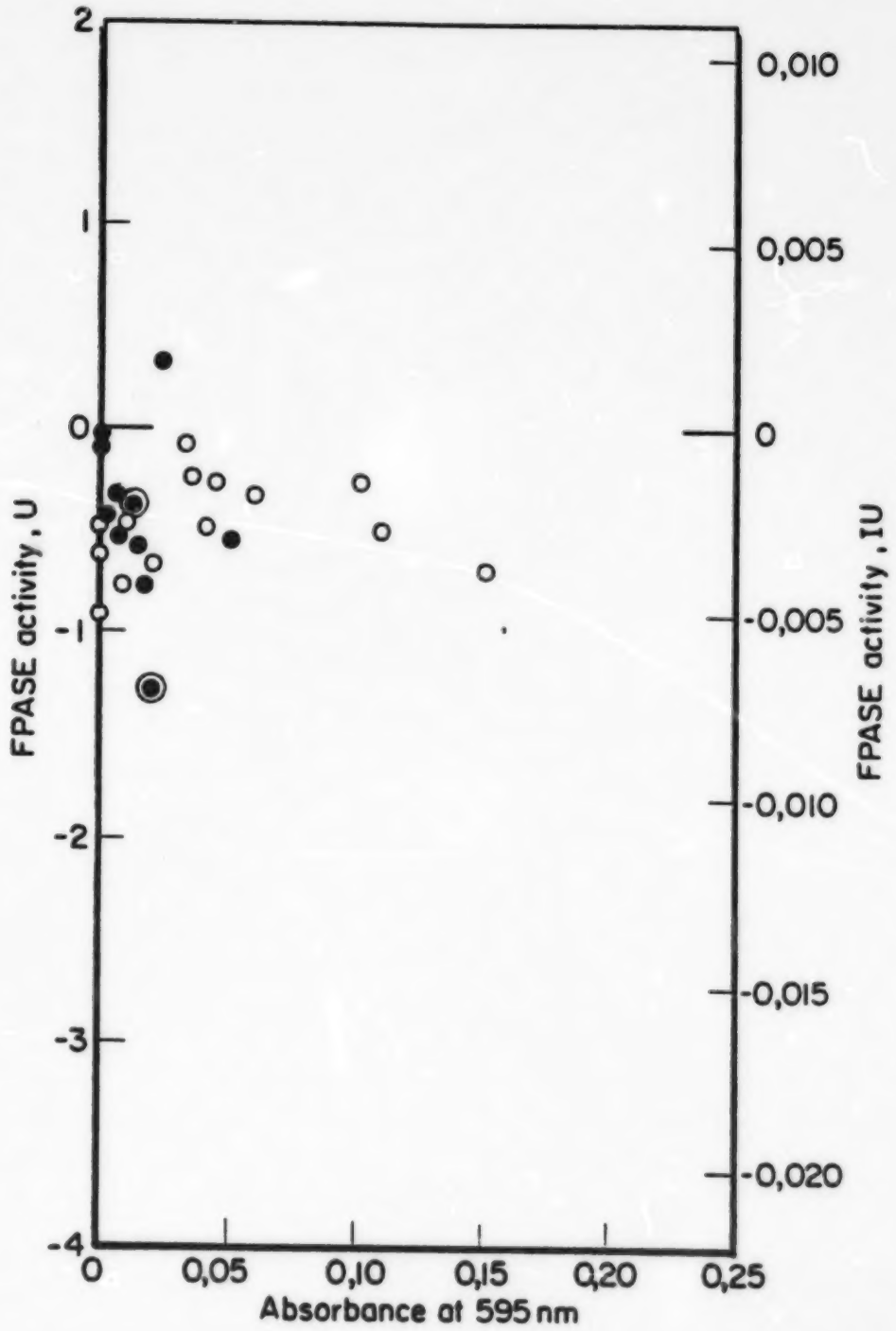


Fig. 3.14 - Mutation tests with X-ray : Relation between FPase activity and released color of survival colonies. Legends: First run (●); Second run (o); Colonies at time zero (◉ ; ◎).

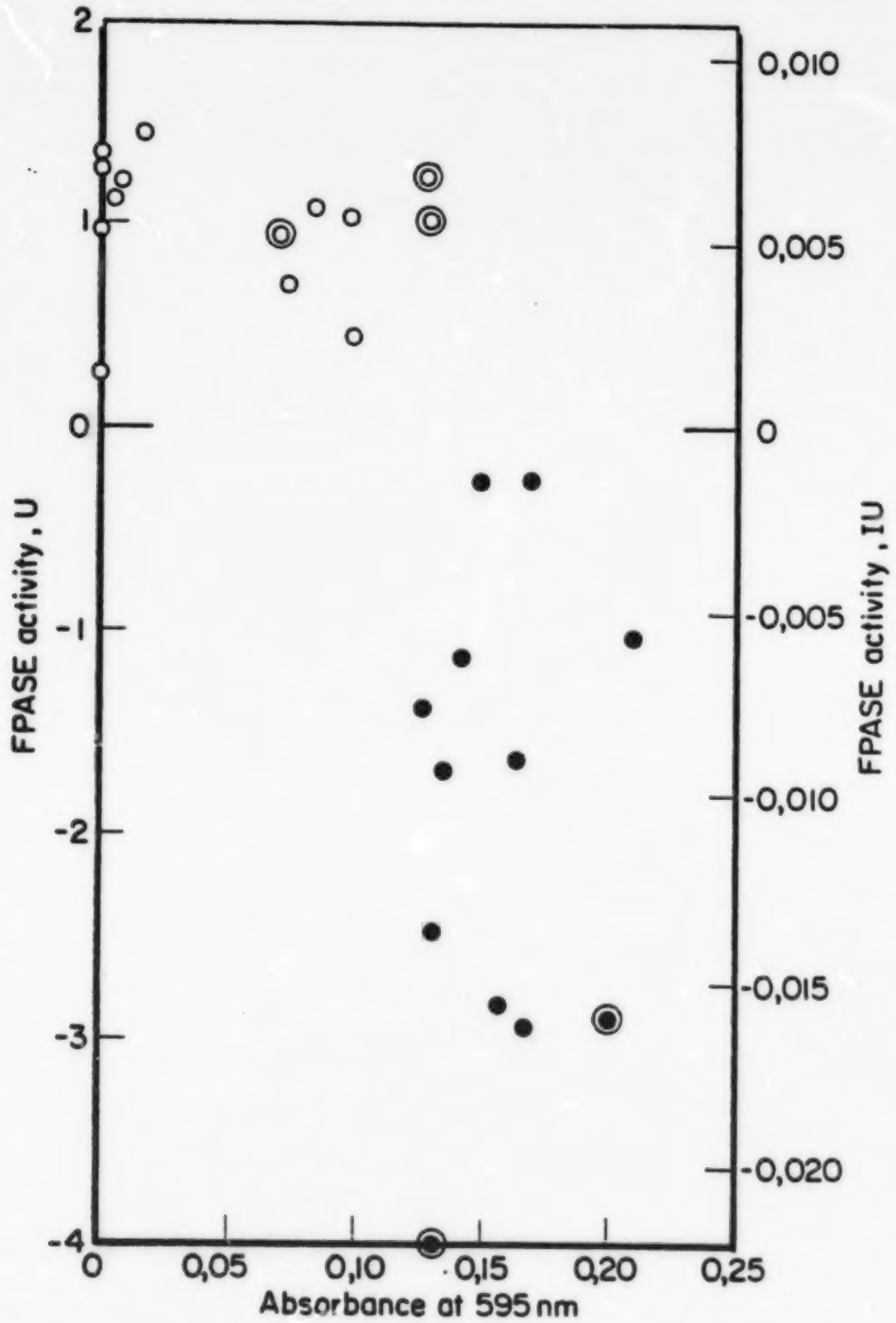


Fig. 3.15 - Mutation tests with EMS : Relation between FPase activity and released color of survival colonies. Legends: First run (●); Second run (o); Colonies at time zero (◉ ; ◎).

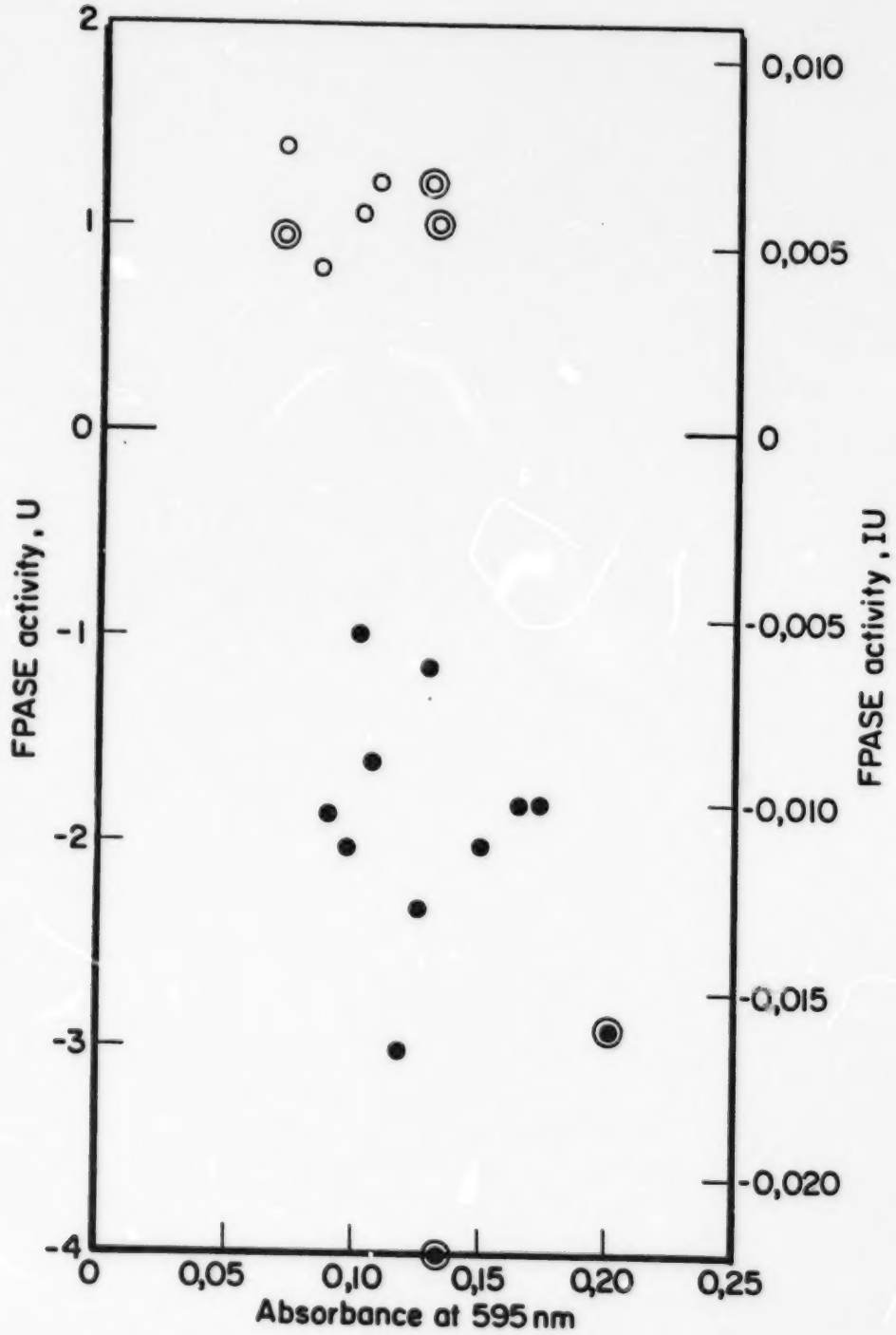


Fig. 3.16 - Mutation tests with NTG : Relation between FPase activity and released color of survival colonies. Legends: First run (●); Second run (o); Colonies at time zero (◉ ; ◎).

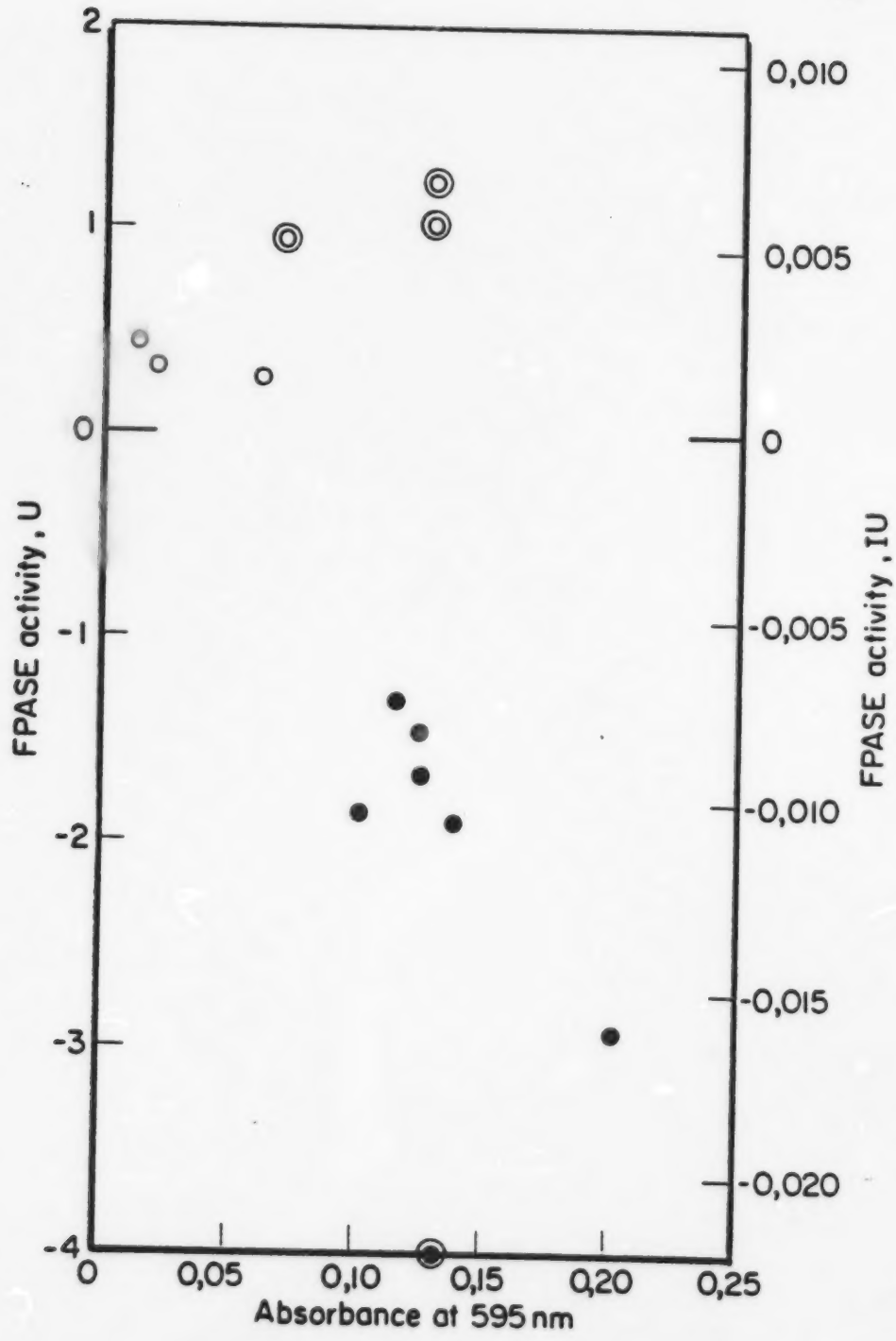


Fig. 3.17 - Mutation tests with nitrous acid : Relation between FPase activity and released color of survival colonies. Legends: First run (●); Second run (o); Colonies at time zero (◉ ; ◎).

low levels of FPase activity (<0,01 IU) and of dye precipitation under acidic solution (culture medium filtrate at pH 2,0).

3.3 ON THE KINETICS OF FERMENTATION IN BIOREACTORS

3.3.1 Batch fermentation

During the 10-day period of batch fermentation in bioreactors, pH was self-stabilized at 2,01 ± 0,02; temperature was maintained most of the time between 27-28 °C and DO concentration was always superior to 6,4 mg/ℓ (Figures 3.18 and 3.19; Table B.11). The results from two pairs of bioreactors - one with newspaper substrate, another with CMC-xylan substrate- were almost identical, thus indicating good reproducibility of fermentation conditions.

ATP concentration in newspaper medium (Figure 3.20; Table B.12) was gradually increased to around 0,4 mg/ℓ at 80-100 h and stayed at this value to the end of fermentation. In CMC-xylan medium, ATP concentration exponentially reached a maximum value of around 2,0 mg/ℓ at 80 h of fermentation and then oscillatorily decreasing to approximately 1,1 mg/ℓ at the end of fermentation. Such oscillatory phenomenon was not observed with newspaper medium, probably because of its very low values of ATP concentration. A lag phase of approximatively 15 h was observed in both media. The variation of ATP concentration in the two pairs of bioreactors followed the same pattern and reproducibility was fairly reasonable.

The level of ATP concentration in the CMC-xylan medium (Figure 3.21) was three-to-five fold of that in the newspaper medium. It was to be expected, therefore, that cell growth was more abundant in the CMC-xylan medium.

Biomass concentration in CMC-xylan medium (Figure 3.22; Table B.13) decreased during the lag phase period of ATP concentration.

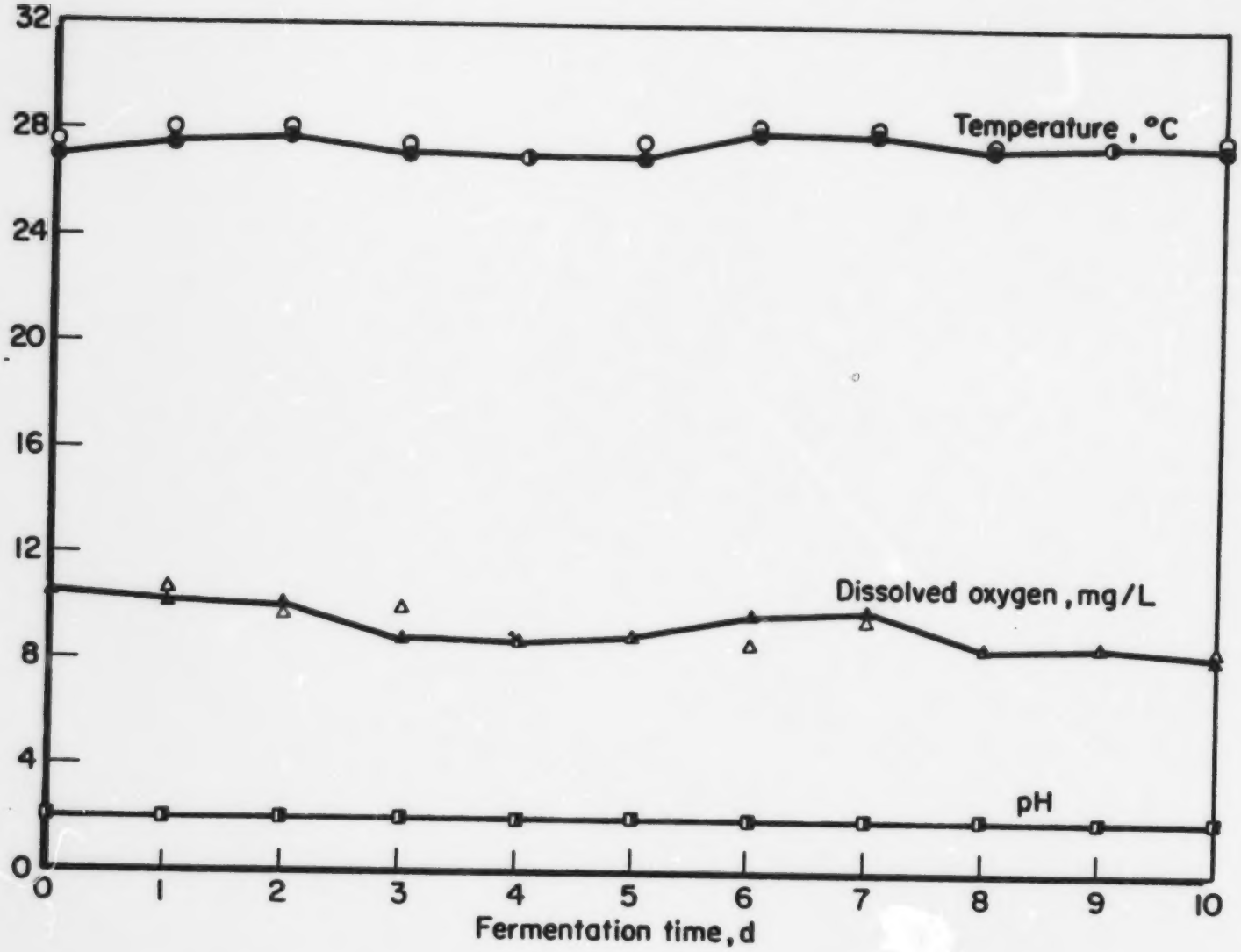


Fig. 3.18 - Batch fermentation in bioreactors: Variations of pH, temperature and DO concentration in newspaper medium.
Legends: Bioreactor 1A (o, △, □); Bioreactor 1B (●, ▲, ■).

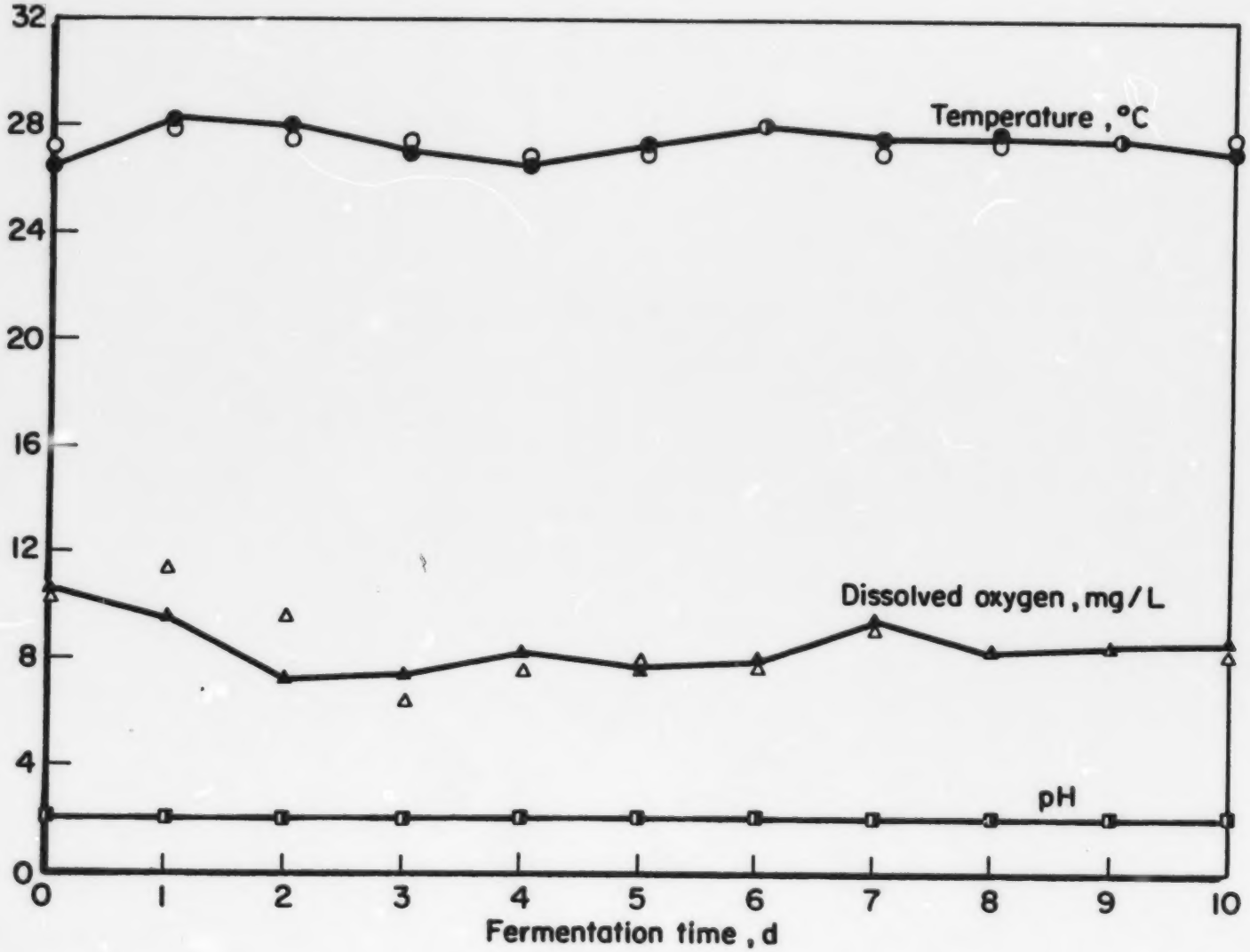


Fig. 3.19 - Batch fermentation in bioreactors: Variations of pH, temperature and DO concentration in CMC-xylan medium.
Legends: Bioreactor 2A (o, △, □); Bioreactor 2B (●, ▲, ■).

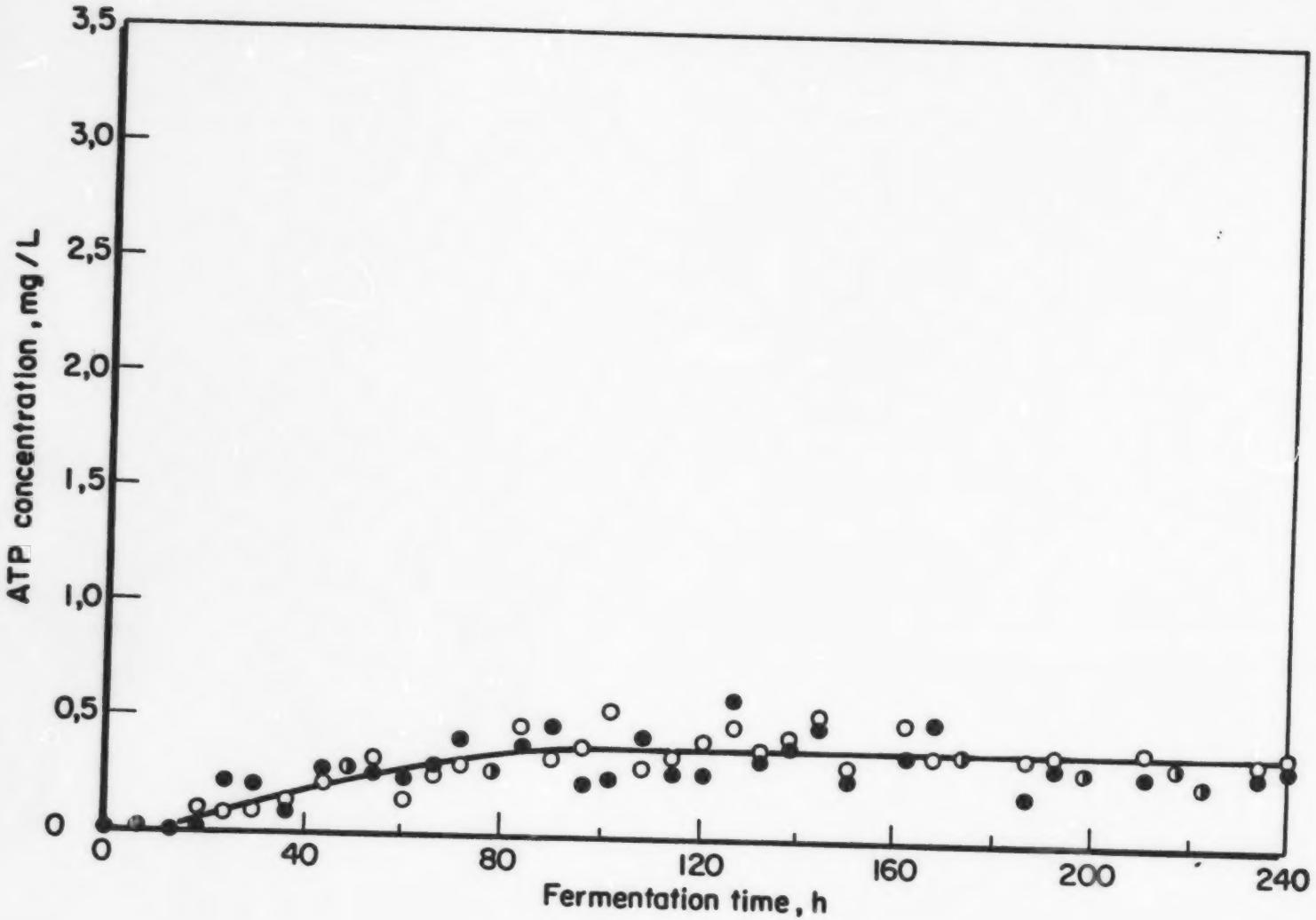


Fig. 3.20 - Batch fermentation in bioreactors: Variation of ATP concentration in newspaper medium.
Legends: Bioreactor 1A (o); Bioreactor 1B (●).

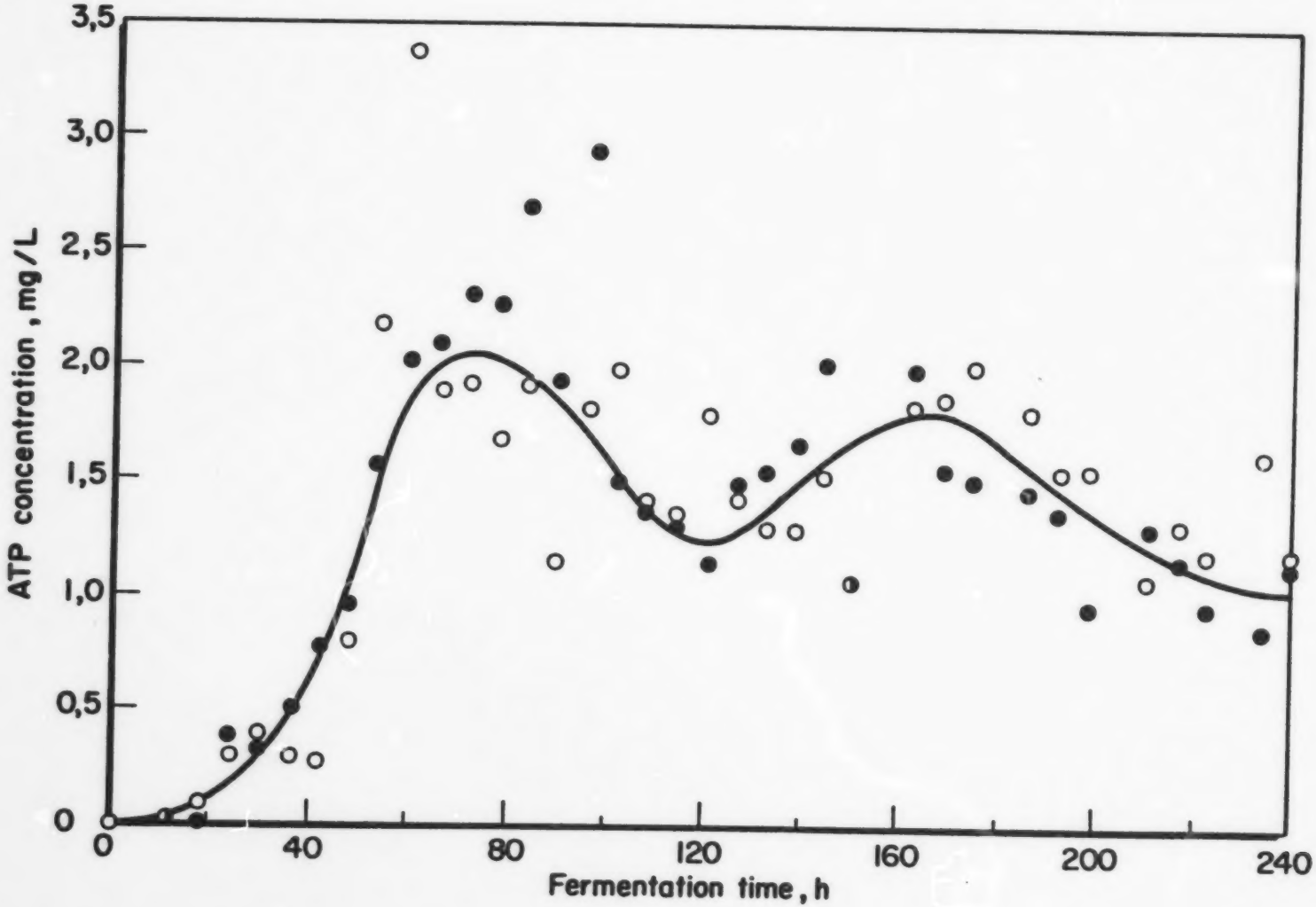


Fig. 3.21 - Batch fermentation in bioreactors: Variation of ATP concentration in CMC-xylan medium.
Legends: Bioreactor 2A (o); Bioreactor 2B (●).

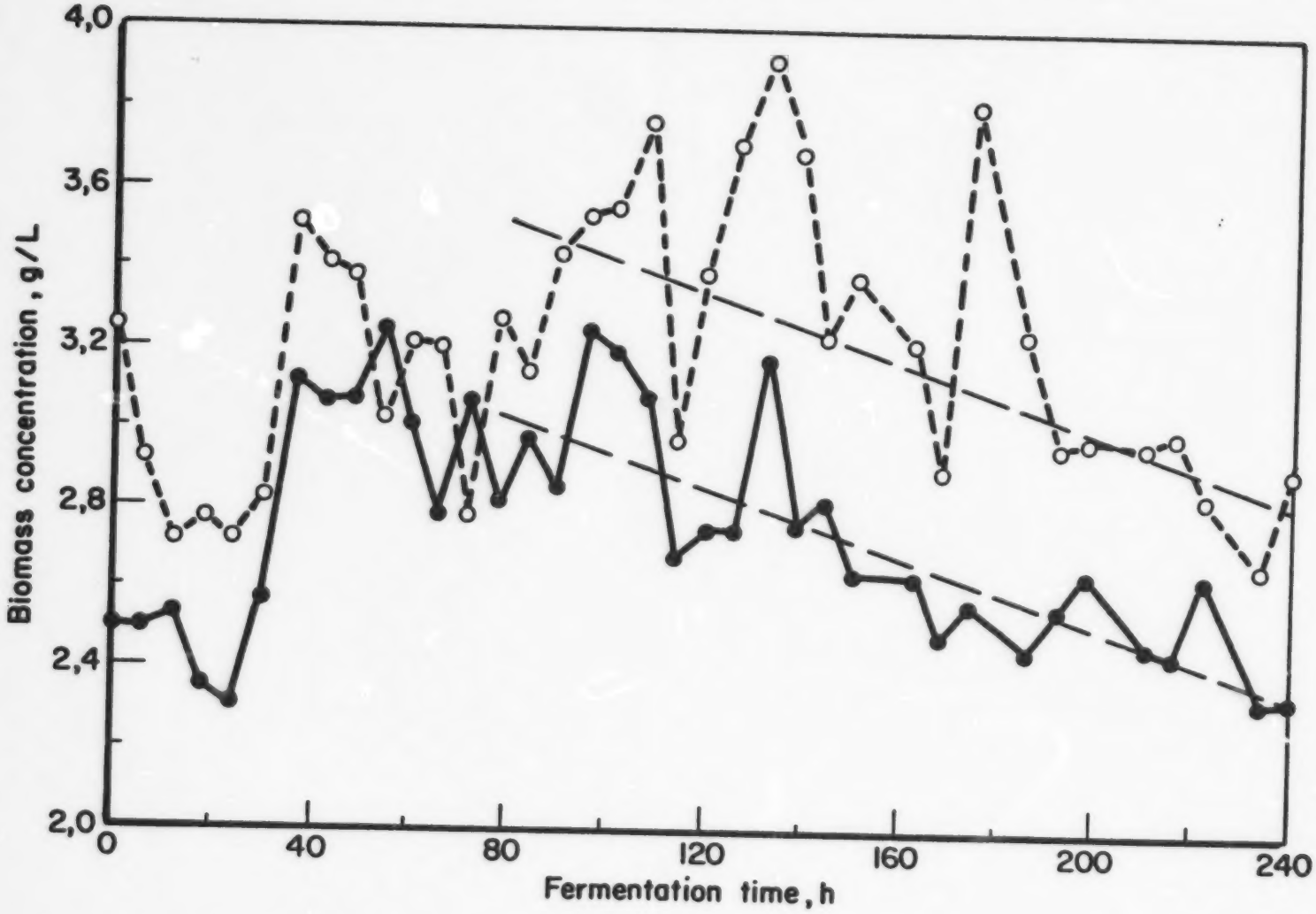


Fig. 3.22 - Batch fermentation in bioreactors: Variation of biomass concentration in CMC-xylan medium.
Legends: Bioreactor 2A (o); Bioreactor 2B (●).

Then, it increased rapidly to 3,1 - 3,4 g/ℓ in concordance with the exponential phase at ATP concentration, and gradually decreased again with a rate of approximately 0,1 g/ℓ-d until the end of fermentation. The two curves from the pair of bioreactors followed the same pattern; however, the fluctuation of experimental data was very high. No apparent periodic oscillation was observed from biomass concentration curves after 80 h as it was for ATP concentration curves during the same period.

ATP concentration seemed to follow biomass concentration in CMC-xylan medium in different phases of the growth curve in batch fermentation kinetics.

The ATP pool in CMC-xylan medium (Figure 3.23; Table B.13) exponentially increased to a maximum value of 0,7 - 0,9 mg ATP/g biomass in concordance with the exponential phase of ATP concentration curves. Afterwards, it fluctuated around 0,5 ± 0,2 mg ATP/g biomass.

Xylanase activity of the culture filtrate in the newspaper medium was very low (below 0,05 IU). Only a small peak of 0,10 - 0,15 IU of xylanase activity was observed at approximately 40 - 60 h of fermentation (Figure 3.24; Table B.14). In the CMC-xylan medium, xylanase activity of the culture filtrate increased rapidly to a maximum value of 1 IU at 60 - 80 h and then stayed constant at this value to the end of batch fermentation (Figure 3.25; Table B.14).

The small peak of xylanase activity observed at 40 - 60 h in newspaper medium (Figure 3.24) did not seem to coincide with any similar phenomenon on the ATP concentration curve (Figure 3.20). However, in CMC-xylan medium, the exponential phase of ATP concentration and the time at its maximum value (Figure 3.21) corresponded quite well to those of the xylanase activity curve (Figure 3.25). Indeed, it was

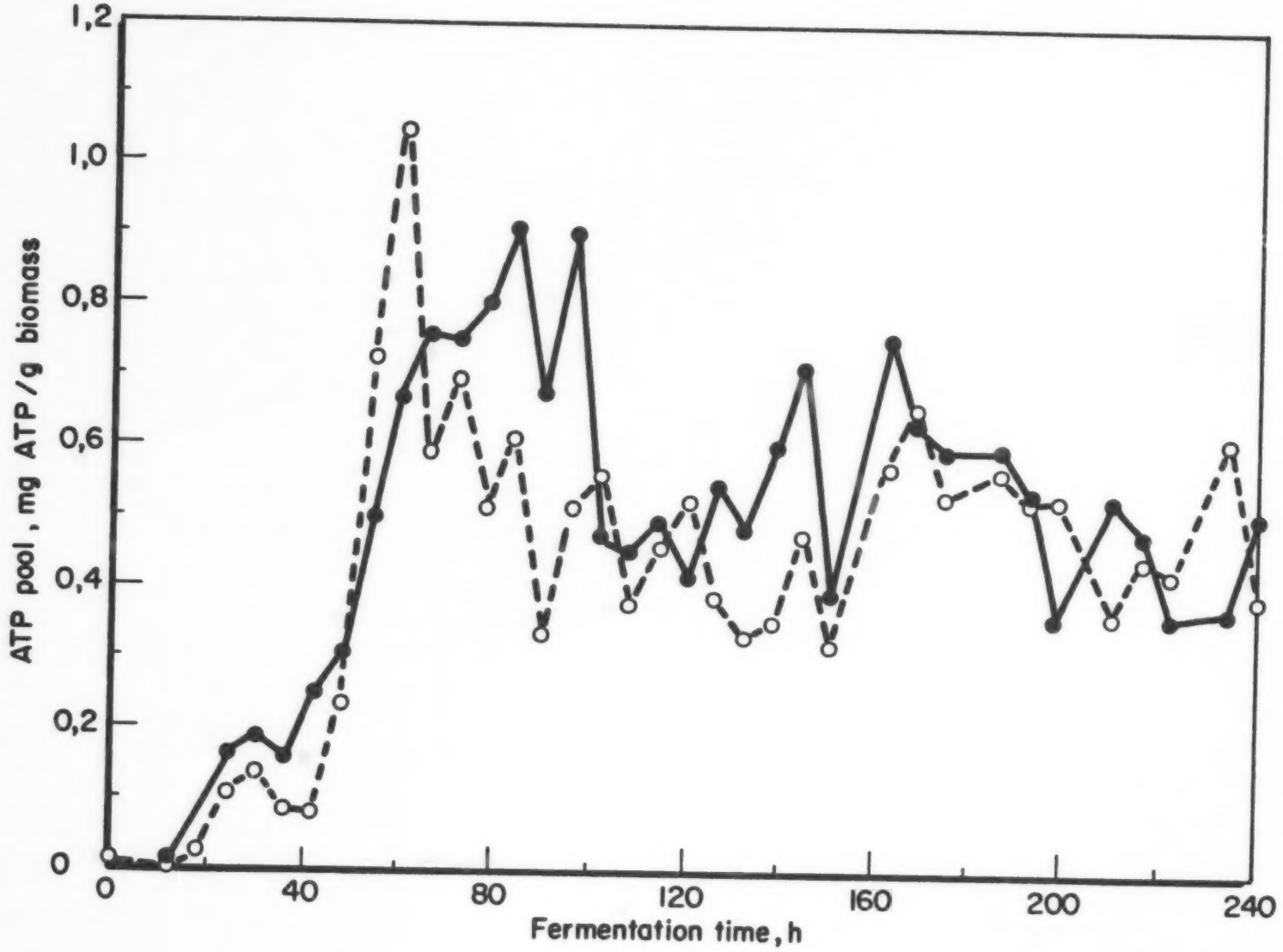


Fig. 3.23 - Batch fermentation in bioreactors: Variation of ATP pool in CMC-xylan medium. Legends: Bioreactor 2A (o); Bioreactor 2B (●).

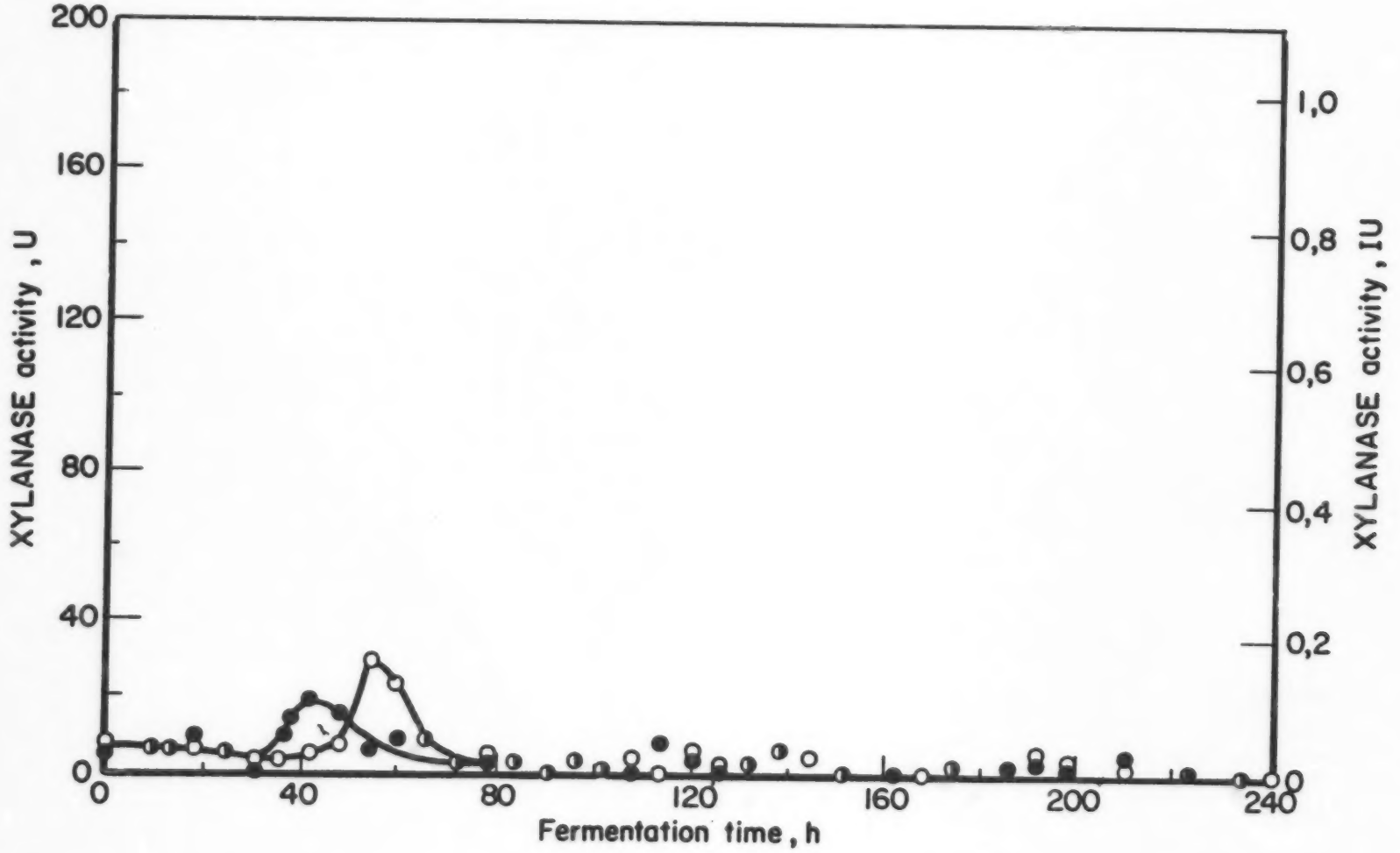


Fig. 3.24 - Batch fermentation in bioreactors: Variation of xylanase activity in newspaper medium.
Legends: Bioreactor 1A (o); Bioreactor 1B (●).

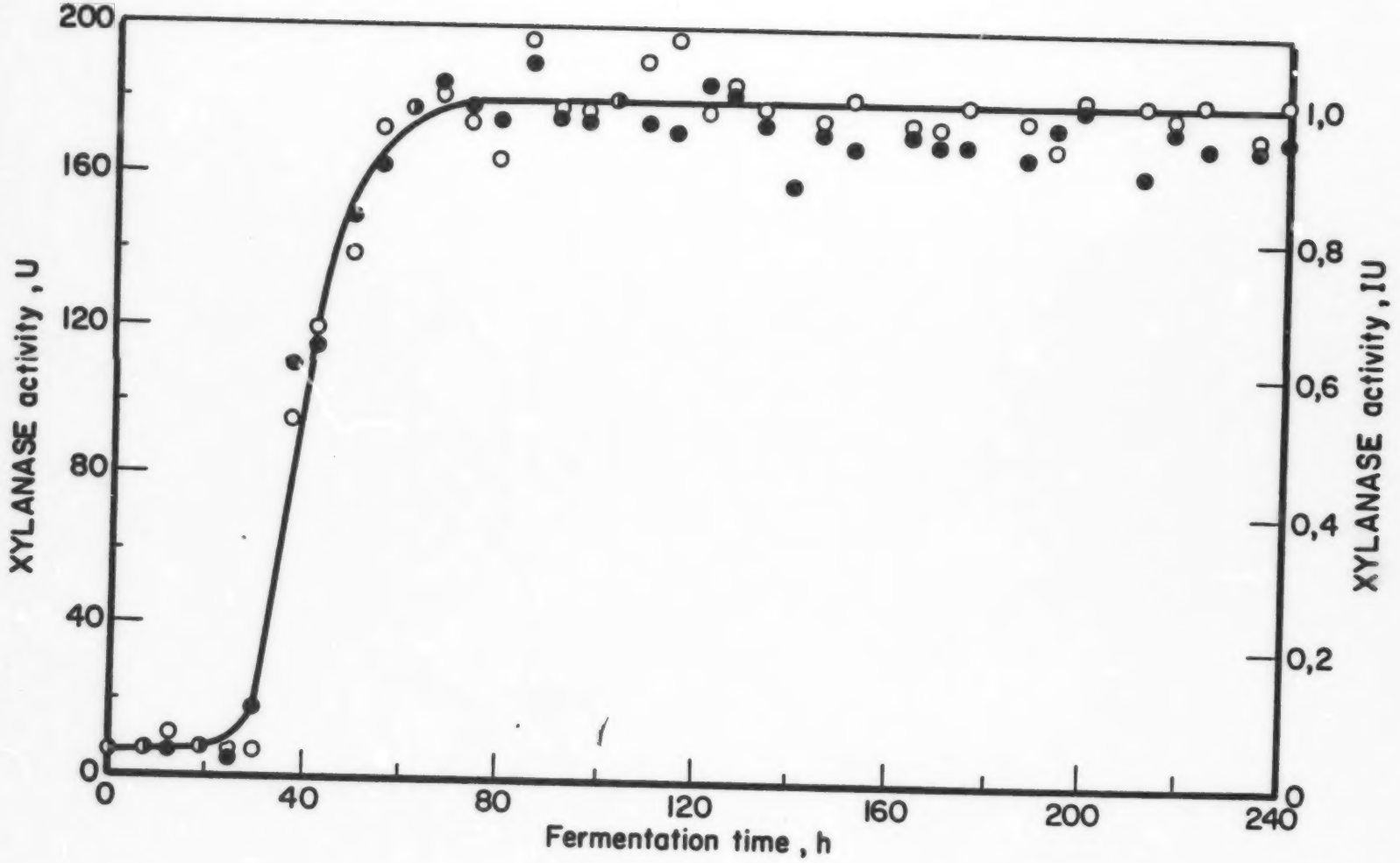


Fig. 3.25 - Batch fermentation in bioreactors: Variation of xylanase activity in CMC-xylan medium.
Legends: Bioreactor 2A (o); Bioreactor 2B (●).

found that xylanase production in CMC-xylan medium is growth associated during the exponential growth phase (Table 3.7), according to this relation:

$$\frac{d[\text{xylanase activity}]}{dt} = 88{,}84 \; \frac{d[\text{ATP concentration}]}{dt} \qquad (3.1)$$

The xylanase production in CMC-xylan medium seemed also to be in concordance with growth in terms of biomass concentration (Figure 3.22). Xylanase was produced during the exponential growth phase (period from 24 to 40 h) and also during the stationary and/or slightly declining growth phase (period from 40 to 80 h). It was not possible to quantify the relationship between xylanase activity and biomass concentration in CMC-xylan medium because of the highly-fluctuating data on biomass concentration.

The maximum value of ATP pool (Figure 3.23) seemed to occur at the same time as the maximum value of xylanase activity in CMC-xylan medium. The ATP pool is an indication of the physiological state of the cells in bioreactors during fermentation.

CMCase activity in the newspaper medium was also very low (Figure 3.26; Table B.15). It increased to a maximum value of 0,015 IU during the exponential phase of the ATP concentration curve (Figure 3.20). A second maximum value of 0,025 IU was observed at approximately 160 h of fermentation. The same behavior was observed for CMCase activity in CMC-xylan medium, except with higher values for the two maxima: they were 0,12 IU and 0,15 IU respectively (Figure 3.27) The oscillatory behavior of CMCase activity curves (Figures 3.26 and 3.27) after 80 h corresponded very well to the oscillatory behavior of ATP concentration curves during the same period (Figure 3.21). This suggested that CMCase synthesis is strongly ATP-dependent in comparison to xylanase synthesis by acidophilic fungus.

Table 3.7 - Batch fermentation in bioreactors : Kinetic parameters.

Parameter	Case 1[a]	Case 2[b]	Case 3[c]
For growth[d]:			
μ	0,1047	0,1047	0,0680
C_{max}	2,3	2,3	0,5
r^2	0,9730	0,9730	0,9546
For enzyme synthesis[e]:			
α	88,84	9,63	14,24
β	0	0	0
r^2	0,8941	0,9795	0,9434

(a) Xylanase activity = f (ATP concentration) in CMC-xylan medium; t = 20 - 80 h.

(b) CMCase activity = f (ATP concentration) in CMC-xylan medium; t = 20 - 80 h.

(c) CMCase activity = f (ATP concentration) in newspaper medium; t = 20 - 80 h.

(d) Logistic model: $\frac{dC}{dt} = \mu C \left(1 - \frac{C}{C_{max}}\right)$

(e) Luedeking-Piret model: $\frac{dP}{dt} = \alpha \frac{dC}{dt} + \beta C$

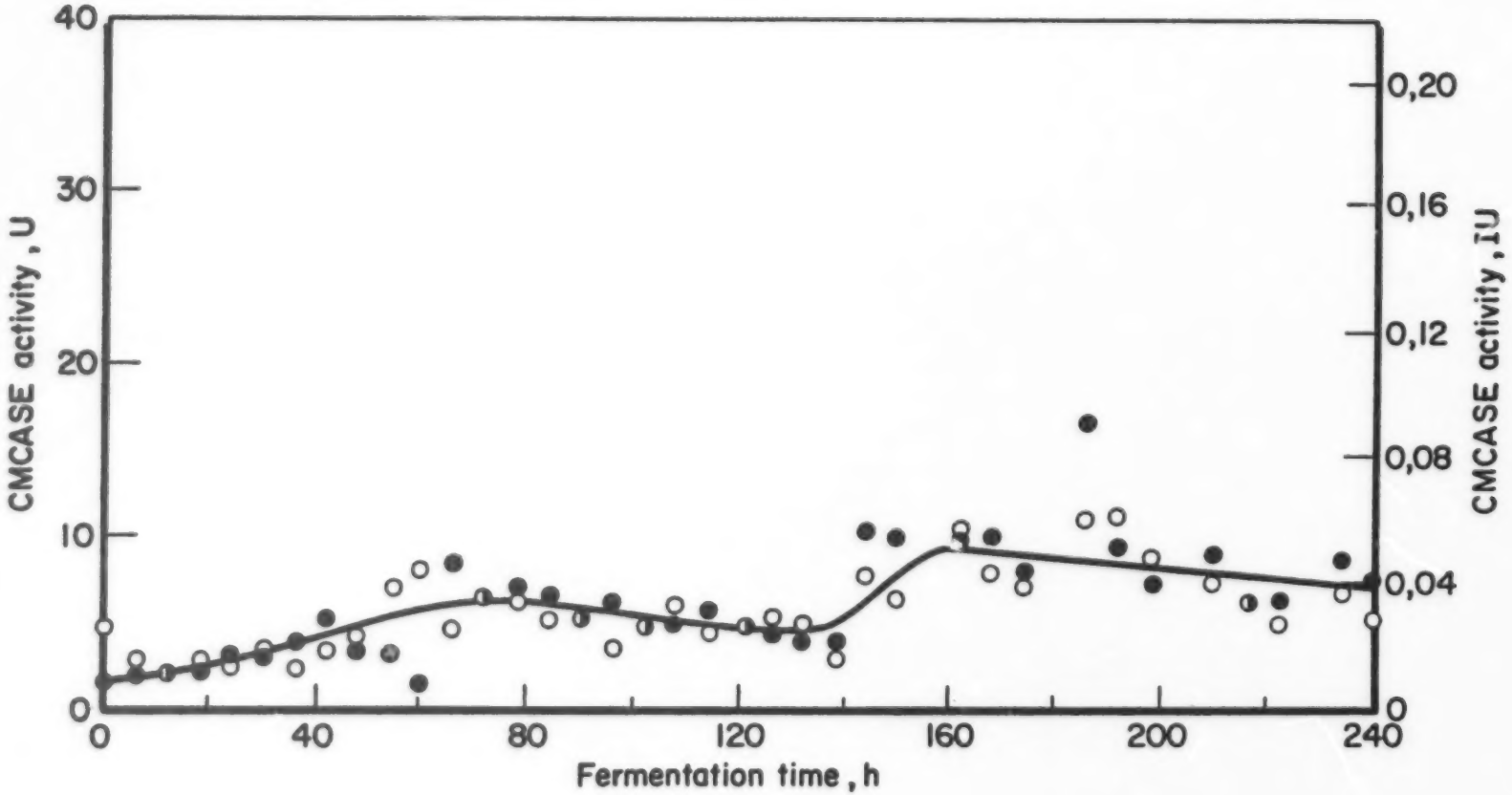


Fig. 3.26 - Batch fermentation in bioreactors: Variation of CMCase activity in newspaper medium.
Legends: Bioreactor 1A (o); Bioreactor 1B (●).

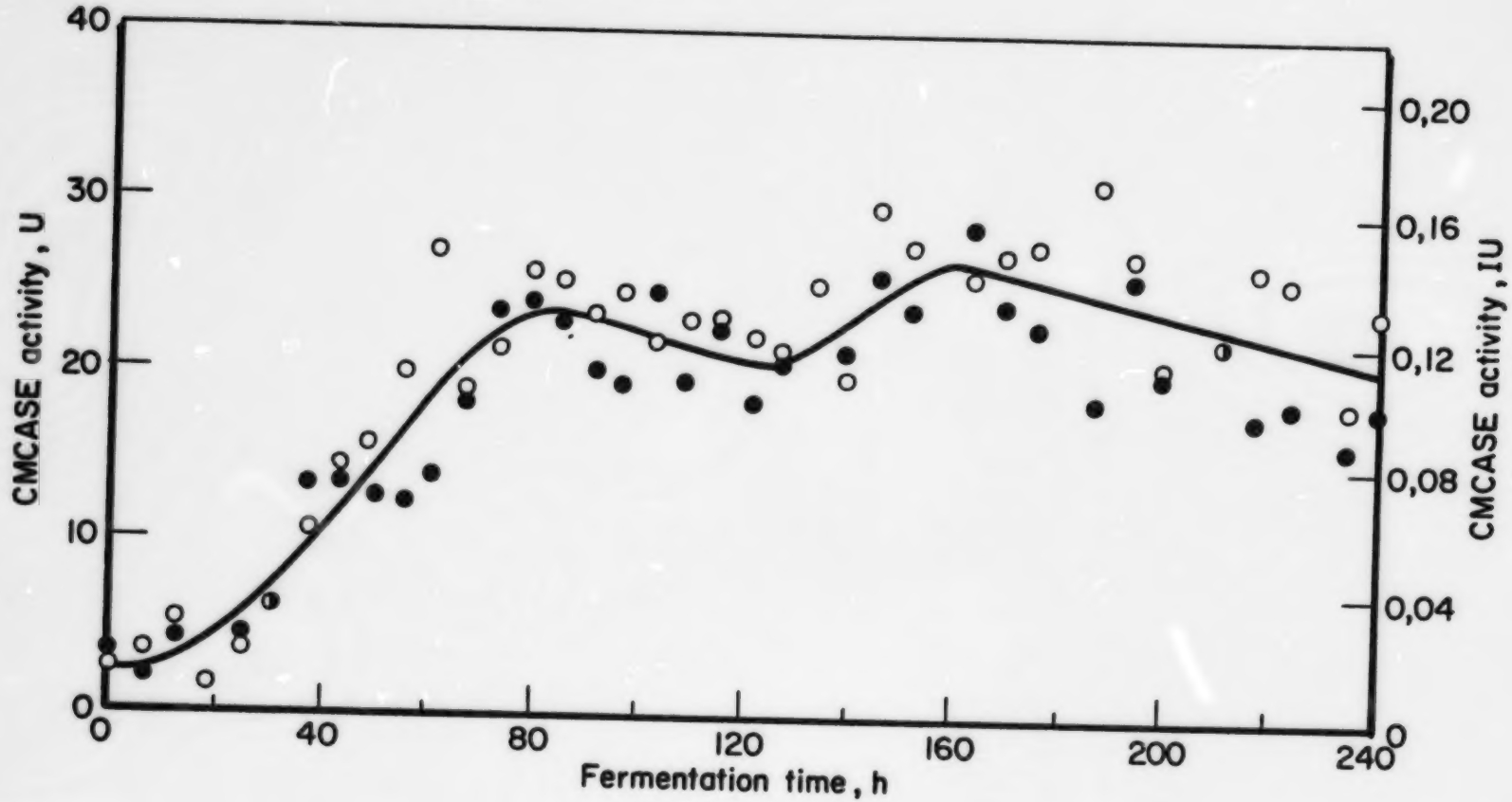


Fig. 3.27 - Batch fermentation in bioreactors: Variation of CMCase activity in CMC-xylan medium.
Legends: Bioreactor 2A (o); Bioreactor 2B (●).

It was found that the production of CMCase is growth associated in both newspaper and CMC-xylan media during the exponential growth phase, according to the following relations (Table 3.7):

$$\frac{d[\text{CMCase activity}]}{dt} = 9{,}63 \frac{d[\text{ATP concentration}]}{dt} \qquad (3.2)$$

for CMC-xylan medium, and

$$\frac{d[\text{CMCase activity}]}{dt} = 14{,}24 \frac{d[\text{ATP concentration}]}{dt} \qquad (3.3)$$

for newspaper medium

The CMCase activity-ATP concentration ratio (Table 3.8) seemed interestingly constant at around 0,10 IU per mg ATP per mℓ for the period from 40 h to 240 h. This corresponded to the stationary and declining phases of the biomass curve (Figure 3.22). During the biomass growth phase (from 20 to 40 h), higher values were obtained for the CMCase activity-ATP concentration ratio.

In other words, for the synthesis of each IU of CMCase during the stationary and declining growth phase, it required around 10 mg ATP of living cell equivalent/mℓ in the medium. During the growth phase, however, less ATP concentration (down to 5 mg ATP/mℓ) was required for the same purpose. The results suggested that the amount of ATP for CMCase synthesis and growth is less than that required for CMCase synthesis and cell maintenance.

FPase activity was very low (inferior to 0,02 IU) in both newspaper and CMC-xylan media (Figure 3.28; Table B.16).

FPase activity of the culture filtrate did not result from a simple sum of its xylanase and CMCase constituents. This is because the beginning of FPase synthesis and the maximum value of FPase activity did not coincide with the starting points and the maxima of xylanase and CMCase activity curves.

Table 3.8 - Batch fermentation in bioreactors : Variation of CMCase activity - ATP concentration ratio[a].

Fermentation time (h)	CMCase activity : ATP concentration (IU-L/mg)	
	Newsprint medium	CMC-xylan medium
20	0,20	0,20
30	0,15	0,16
40	0,10	0,11
80	0,09	0,06
120	0,06	0,09
160	0,13	0,08
200	0,11	0,10
240	0,10	0,11

(a) Calculated from the average values of CMCase activity (solid curve of Figs. 3.26 and 3.27) and ATP concentration (solid curve of Figs. 3.20 and 3.21).

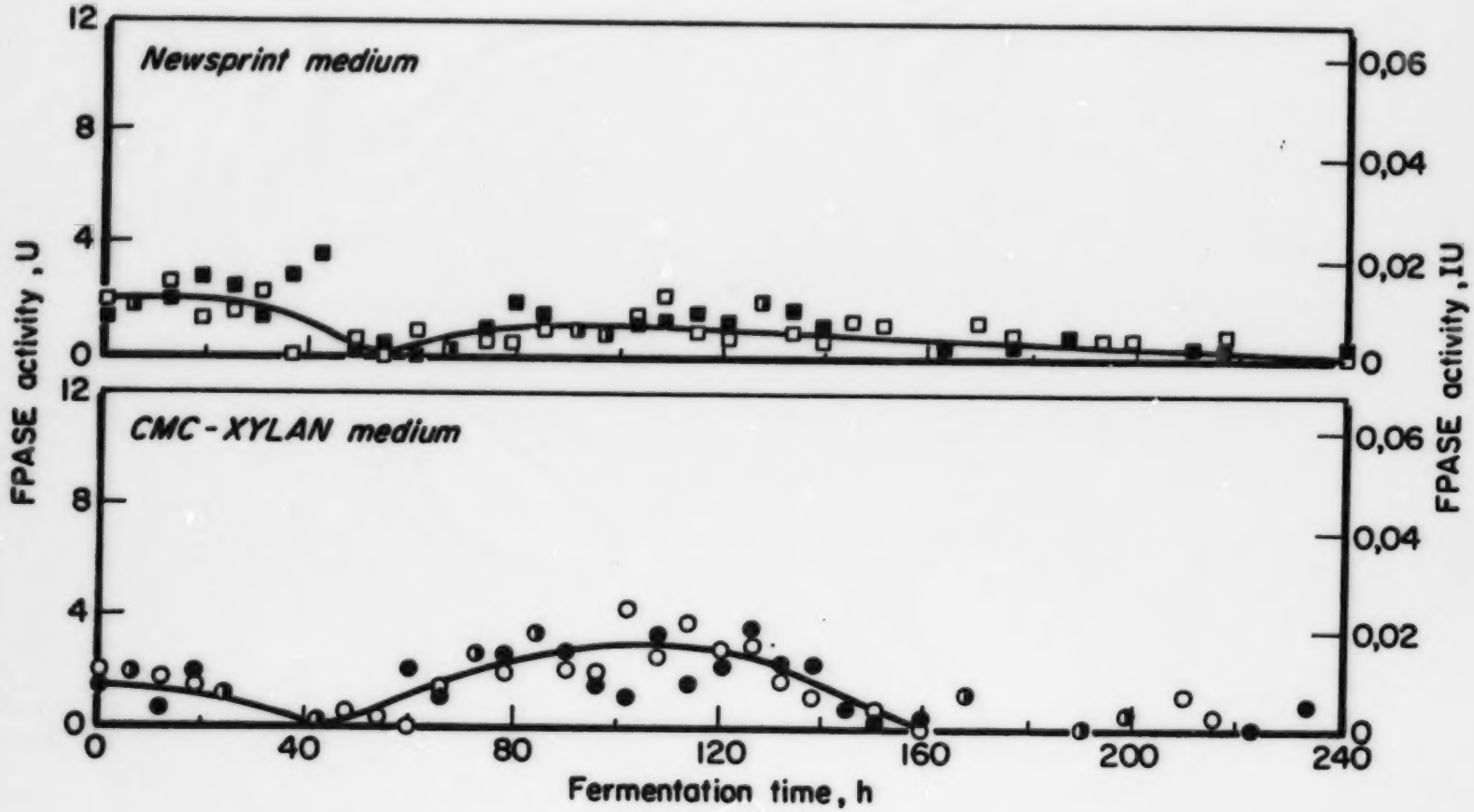


Fig. 3.28 - Batch fermentation in bioreactors: Variation of FPase activity.
Legends: Bioreactor 1A (□); Bioreactor 1B (■); Bioreactor 2A (o);
Bioreactor 2B (●).

FPase activity did not depend on xylanase activity. We can say this because some FPase activity was found in newspaper medium where xylanase activity was absent (Figures 3.24 and 3.28). FPase activity seemed to follow the first cycle of CMCase activity with a delay of approximately 20 hours, and this was observed for both newspaper and CMC-xylan media. The maximum FPase- maximum CMCase (of the first cycle, Figures 3.26 and 3.27) ratio was found to be approximately 0,13 - 0,14 in both media.

Protein concentration of the culture filtrate (free protein) in the newspaper medium was very low (Figure 3.29; Table B.17). It stayed fairly constant around 10 mg/ℓ. This explained the very low enzymatic activities (xylanase, CMCase and FPase) of the culture filtrate observed from the newspaper medium. In the CMC-xylan medium, protein concentration increased exponentially to a peak value of around 40 mg/ℓ at 80 h, then decreased to around 16 mg/ℓ and stayed around this value until the end of fermentation (Figure 3.30). Although the peak of protein concentration was reached at the same time as that of the CMCase activity (first cycle), it seemed that this protein was not composed entirely of cellulolytic enzymes. Otherwise, it is not possible to explain the presence of higher cellulolytic activities (CMCase, FPase) at lower protein concentrations (after 80 h).

If one assumes that the constant base values of protein concentration of around 10 mg/ℓ and 16 mg/ℓ were those of cellulolytic enzymes in the newspaper and CMC-xylan media respectively, maximal specific activity of cellulolytic enzymes could be calculated as reported in Table 3.9. It was found that the specific activities in the newspaper medium were of the same magnitude as those previously reported for the newspaper-xylan medium (Cauchon and Leduy 1985). Specific activities in the CMC-xylan medium were, however, higher than those in the newspaper medium. An interesting observation from Table 3.9 is that the maximum FPase activity-maximum CMCase activity ratio seemed to be a constant of the same magnitude for three different media: 0,10 for the

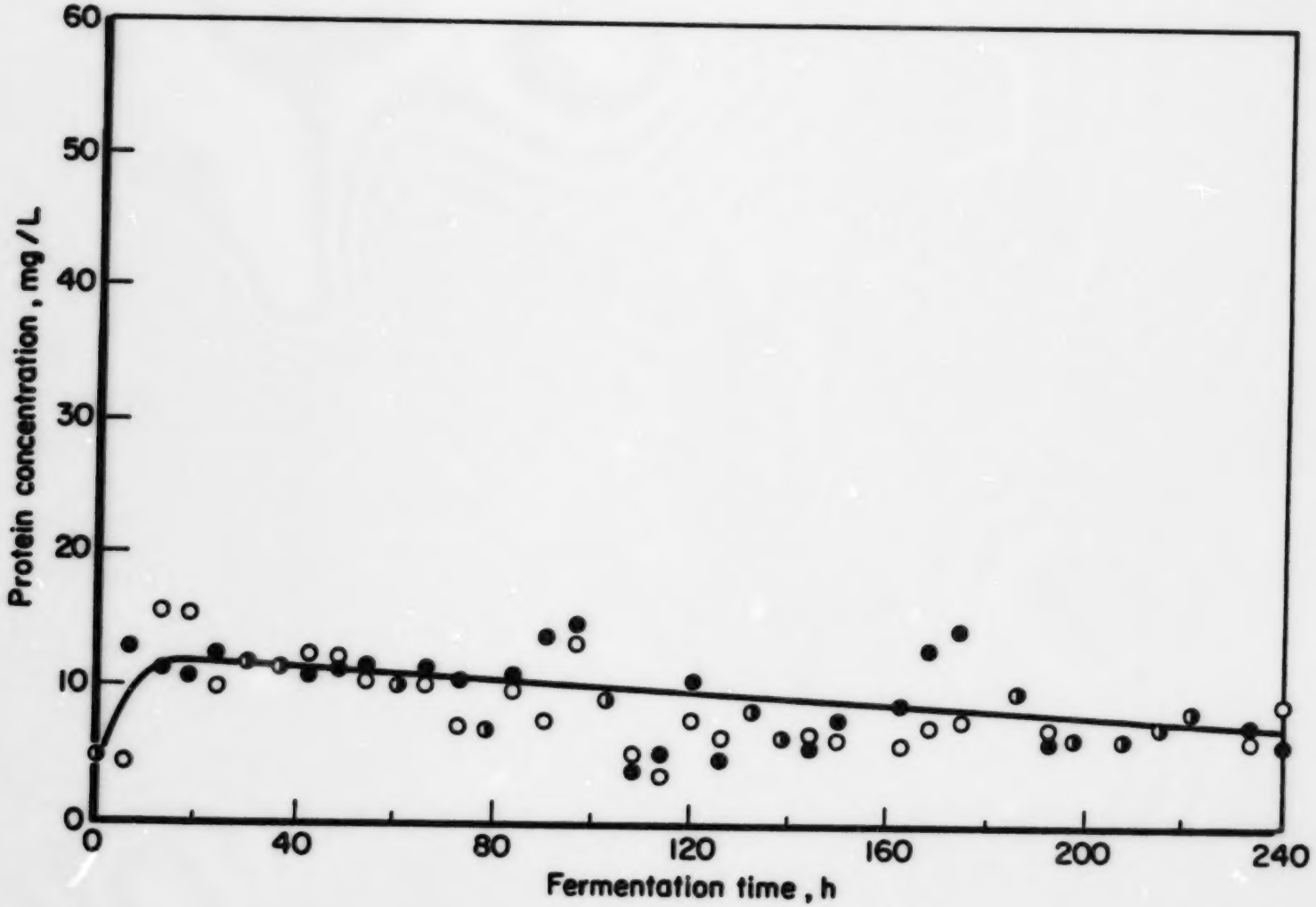


Fig. 3.29 - Batch fermentation in bioreactors: Variation of protein concentration in newspaper medium.
Legends: Bioreactor 1A (o); Bioreactor 1B (●).

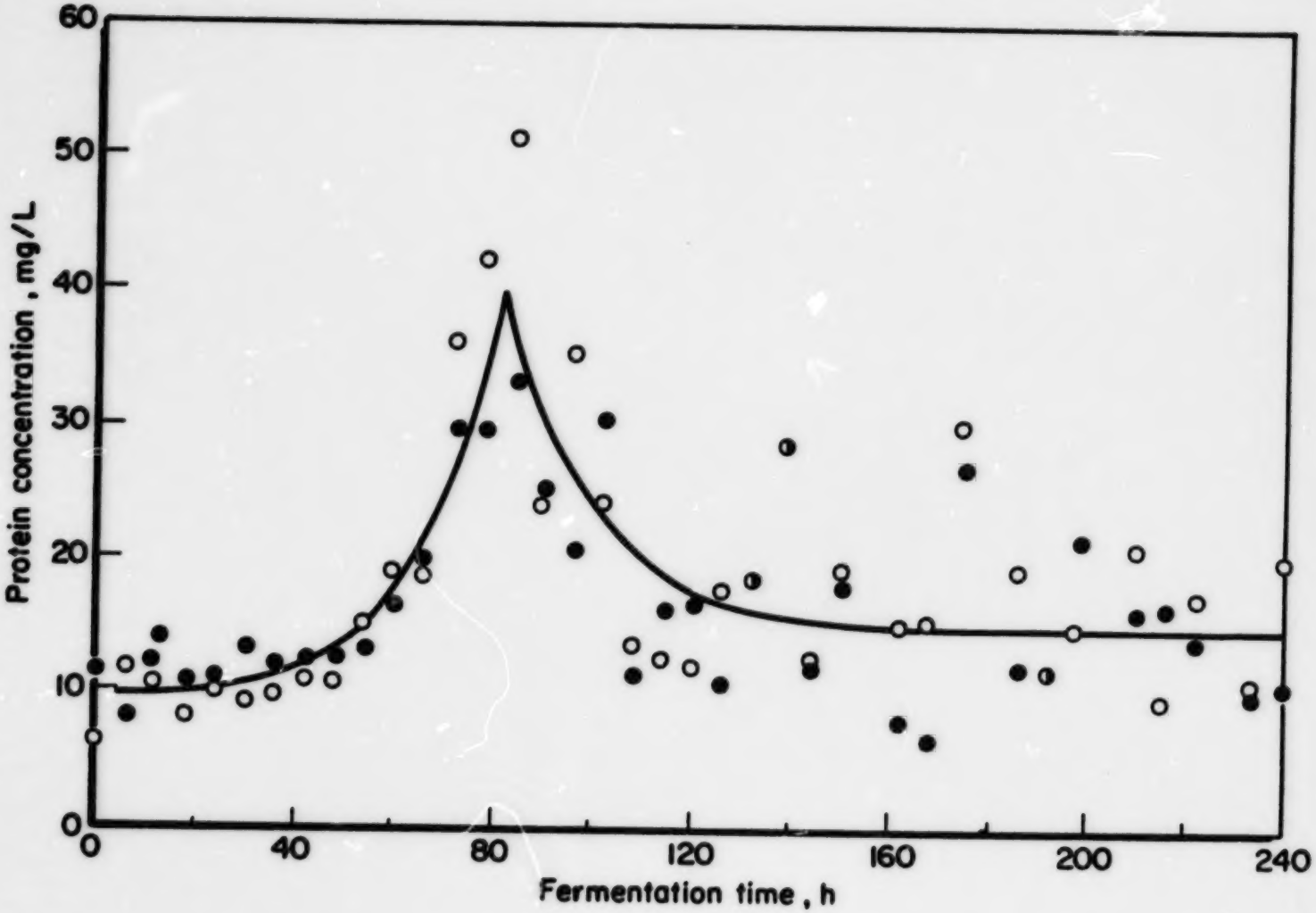


Fig. 3.30 - Batch fermentation in bioreactors: Variation of protein concentration in CMC-xylan medium.
Legends: Bioreactor 2A (o); Bioreactor 2B (●).

Table 3.9 - Batch fermentation in bioreactors : Maximal cellulolytic activities of culture filtrate.

Activity	Volumetric activity[a] (IU)	Protein concentration[b] (mg/mL)	Specific activity (IU/mg protein)
Newsprint medium:			
xylanase	0,15	0,010	15
CMCase	0,05	0,010	5
FPase	0,005	0,010	0,5
CMC-xylan medium:			
xylanase	1,00	0,016	63
CMCase	0,14	0,016	9
FPase	0,02	0,016	1
Newsprint-xylan medium[c]:			
xylanase	1,07	0,148	7,23
CMCase	0,35	0,148	2,36
FPase	0,024	0,148	0,16

(a) From Figs. 3.24 to 3.28.

(b) From Figs. 3.29 to 3.30.

(c) From Cauchon and LeDuy (1985): 14 day-old culture in 5 gallon carboy using 2% w/v milled newsprint and 0,2% w/v xylan as carbon source.

newspaper medium; 0,11 for CMC-xylan the medium and 0,07 for the newspaper-xylan medium.

Volumetric oxygen transfer coefficient k_La rapidly dropped from its initial value of 1 - 2 min^{-1} to around 0,2 min^{-1} (in newspaper medium) or 0,1 min^{-1} (in CMC-xylan medium) at the 24th hour of fermentation. Then, it stayed constant around these values until the end of fermentation (Figures 3.31 and 3.32; Tables B.18 and B.19). Values of k_La of 1 min^{-1} and 0,1 min^{-1} were the same as those found in the culture medium containing ~ 0 and 14 g/ℓ of Penicillium chrysogenum cells respectively (Atkinson and Mavituna 1983). In other types of culture media, values of k_La ranged from 7 min^{-1} at the beginning of fermentation to 1 min^{-1} at the end of fermentation (Atkinson and Mavituna 1983). The turning point of the k_La curves corresponded to the beginning of growth (ATP concentration; Figures 3.20 and 3.21) and enzyme production (CMCase activity; Figures 3.26 and 3.27).

Volumetric rates of oxygen consumption $Q_{O_2}C$ in two different media were similar and seemed to stay constant around values not exceeding 0,1 mg O_2/ℓ-min. These values were very low as compared to the maximum $Q_{O_2}C$ of other fermentations: 0,5 - 1,0 mg O_2/ℓ-min for activited sludge system; 5 - 8 mg O_2/ℓ-min for yeasts; and 10 - 15 mg O_2/ℓ-min for Penicillium chrysogenum (Atkinson and Mavituna 1983). This is because of the very low concentration of acidophilic biomass in the culture medium (not exceeding 4 g/ℓ in CMC-xylan medium, Figure 3.22) and also probably because of the low metabolic activity of the acidophilic fungus.

3.3.2 Continuous fermentation

Several attempts at continuous fermentation for studying growth and cellulolytic enzyme production failed because of the contamination problem in large-scale and long-term operations. Details of the

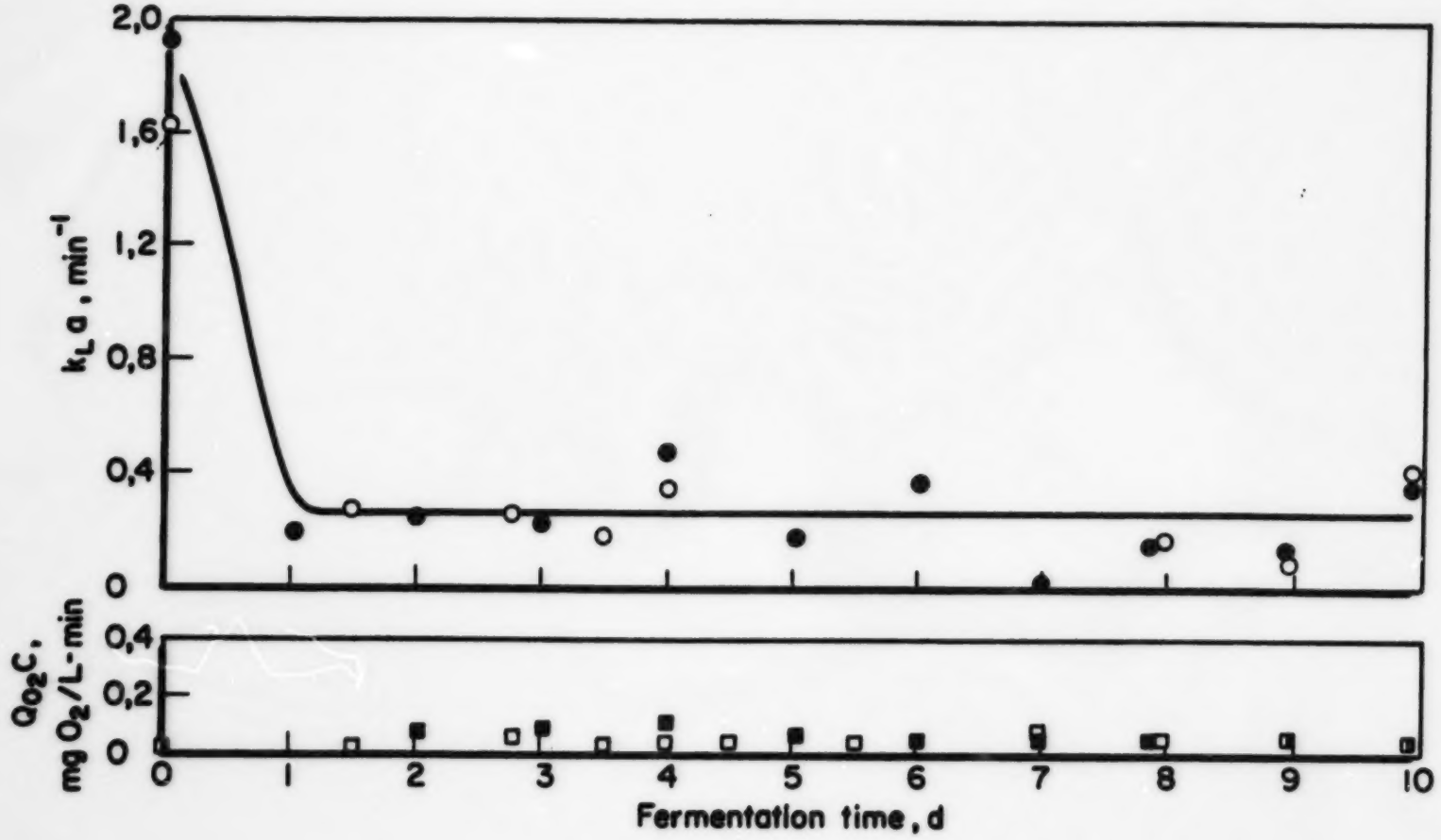


Fig. 3.31 - Batch fermentation in bioreactors: Variations of $k_L a$ and $Q_{O_2}C$ in newspaper medium.
Legends: Bioreactor 1A (o, □); Bioreactor 1B (●, ■).

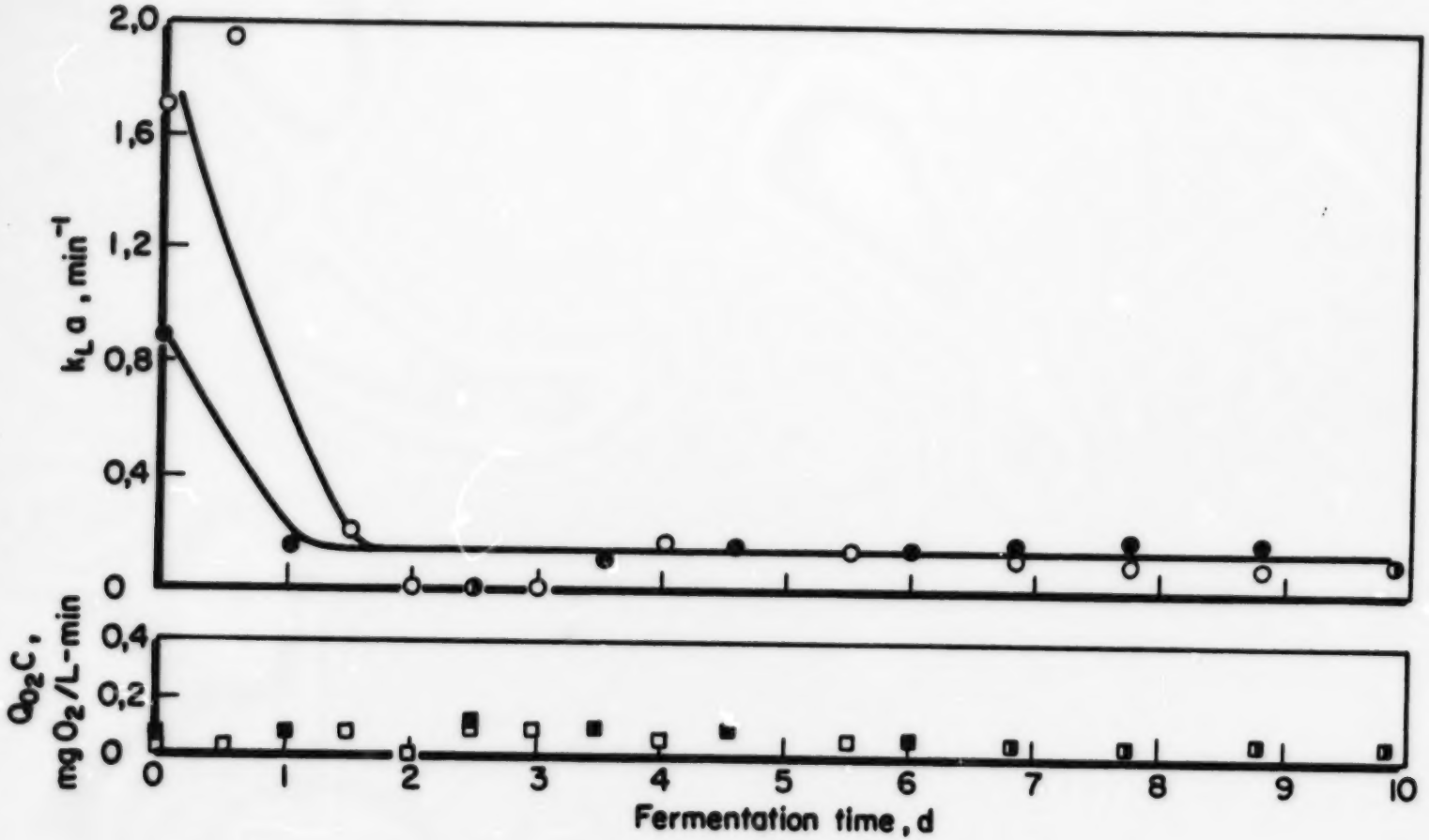


Fig. 3.32 - Batch fermentation in bioreactors: Variations of $k_L a$ and $Q_{O_2}C$ in CMC-xylan medium.
Legends: Bioreactor 2A (o, □); Bioreactor 2B (●, ■).

contamination problem will be discussed in paragraph 3.4. Consequenthy, information (i) on the relationships between Q_{O_2} and specific growth rate μ and specific enzyme production rate ν; (ii) on critical oxygen concentrations for cell growth and for enzyme production; and (iii) on the maintenance coefficient for oxygen Mo could not be obtained. Such information could only be determined from continuous fermentation data (Ryu et al. 1979).

3.4 ON THE CONTAMINATION PROBLEM

3.4.1 Contamination and contaminants

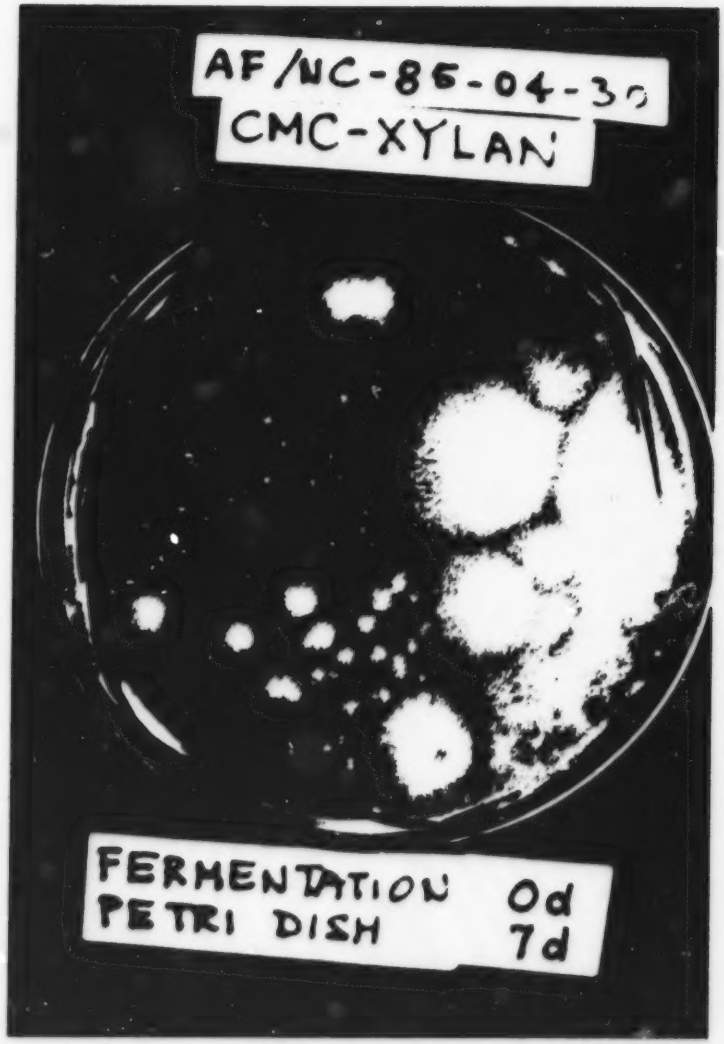
As stated in paragraph 1.3, a nonaseptic and nonsterile process for axenic or pure cultures does not exist. If a desired pure culture is capable of growing and to synthesizing desired products under extreme physiological conditions of pH, temperature, chemical environments, etc., contamination by other species could be eliminated or reduced to an insignificant level. The best (and most severe) environments for testing this hypothesis are microbiology and fermentation laboratories, where many species of microorganisms are present at very high concentrations.

When a pure culture of acidophilic fungus was used as inoculum for nonsterile and nonsapetic experiments in Erlenmeyer, the contamination problem did not seem to exist. Indeed, during the 1982-1984 period, the stock culture of acidophilic fungus for use in the preparation of inoculum had been maintained by periodic transfers in CMC-xylan medium or in glucose medium under nonsterile and non aseptic conditions.

In 1983 and 1984, about a dozen experiments were conducted in 5-gallon carboys containing about 12 ℓ each of either CMC-xylan or malt extract medium for the production of large quantities of acidophilic fungus cells for immobilization on biodics. The contamination problem did not seem to exist during that period because very dense mycelial

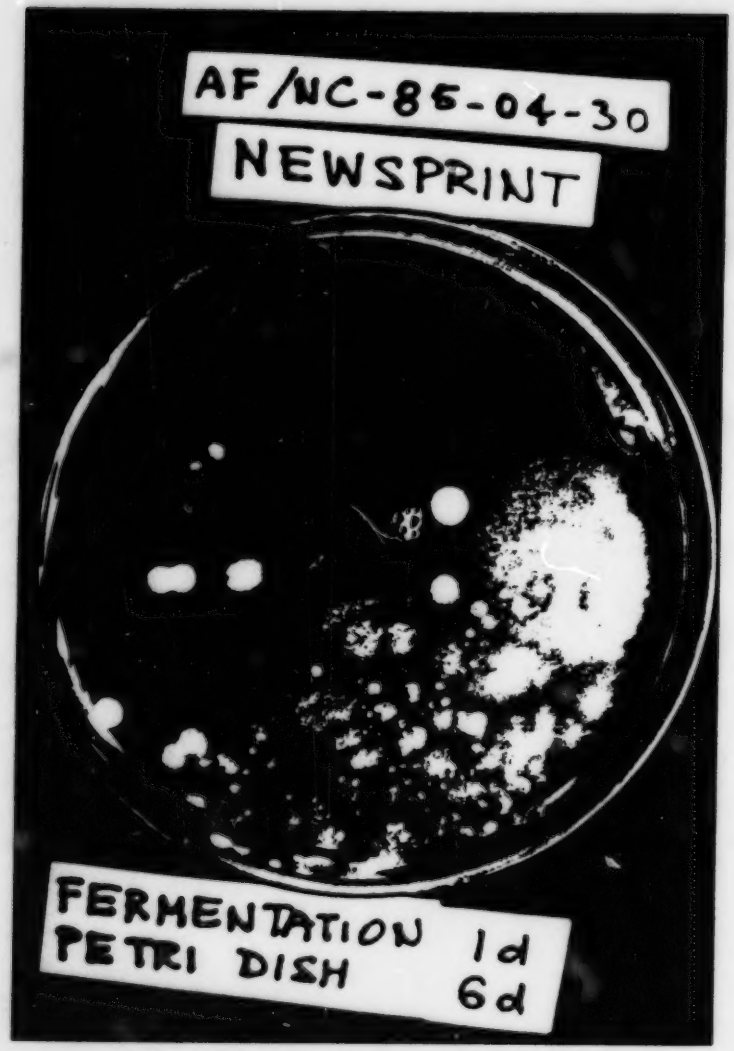
growth was obtained in the culture medium at pH 2,0 after one week, under nonsterile and nonaseptic conditions. Biodiscs prepared with acidophilic fungus cells obtained from carboys, were also successfully operated as open systems (the biodisc bioreactors were not covered) in the laboratory for at least two weeks (LeDuy and Côté 1985). Under such conditions, yeast contaminant appeared in the biodisc bioreactor (in liquid medium) only after two weeks of operation under nonsterile and nonaseptic conditions.

In order to survey contamination level in the bioreactor operation, the batch fermentation experiment in bioreactors (results shown in paragraph 3.3.1) was followed by streaked-plate testing in every step from inoculum preparation to the end of fermentation. The preparation of inoculum for this experiment was done under sterile and aseptic conditions through six transfer steps from stock culture to inoculum growth in 5-gallon carboys (Figure 2.7). The streaked plates showed that pure cultures of acidophilic fungus were obtained at the end of all six transfer steps. Then, the harvesting and washing of the cell growth from the carboys was done under nonsterile and nonaseptic conditions in the laboratory. These operations lasted for two or three days. Then culture media for batch fermentation experiments were inoculated with washed acidophilic fungus cells and conducted under nonsterile and nonaseptic conditions. The streaked plates pictures in Figures 3.33 to 3.38 show that the yeast contaminant (small, white, and smooth colonies) was present in the bioreactor immediately at the beginning of fermentation. This yeast contaminant could have been introduced into the system during several days of harvesting and washing of the inoculum. The yeast contaminant population was significantly increased in the bioreactors after two days of fermentation. This is because of the availability of fermentable sugars in the medium resulting from the action of fungal cellulolytic enzymes on cellulosic substrates. During these batch fermentation experiments, the presence of the yeast contaminant, even in a very significant level toward the end of the fermentation, did not seem to disturb the production of



100

Fig. 3.33 - Microflora in bioreactors immediately after inoculation.



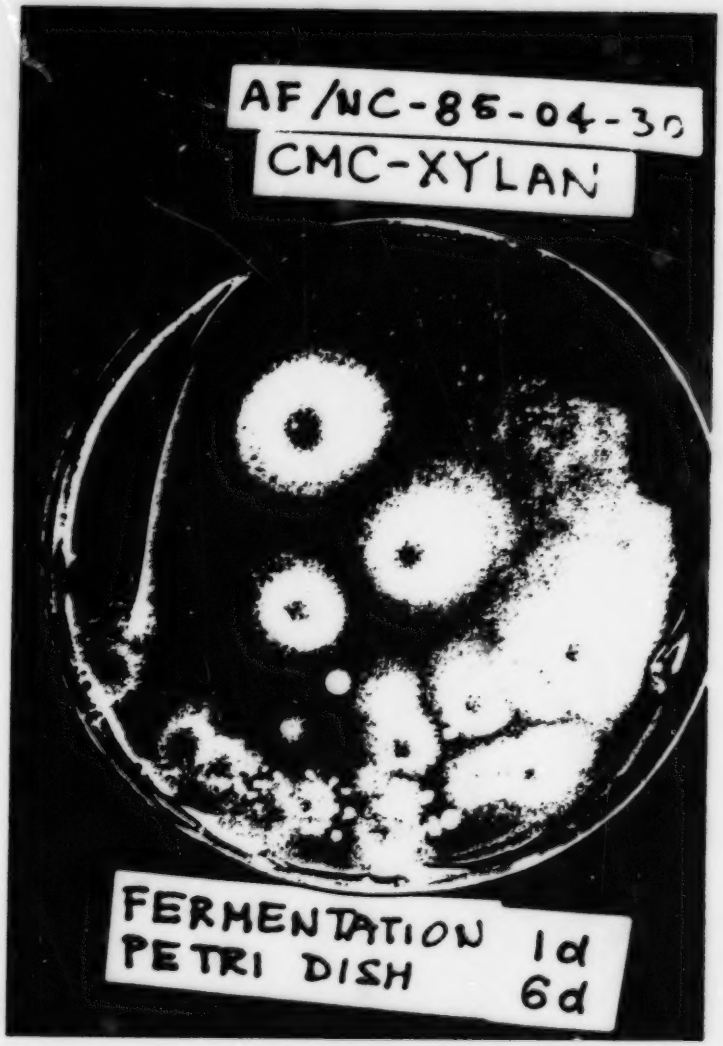


Fig. 3.34 - Microflora in bioreactors on the first day.

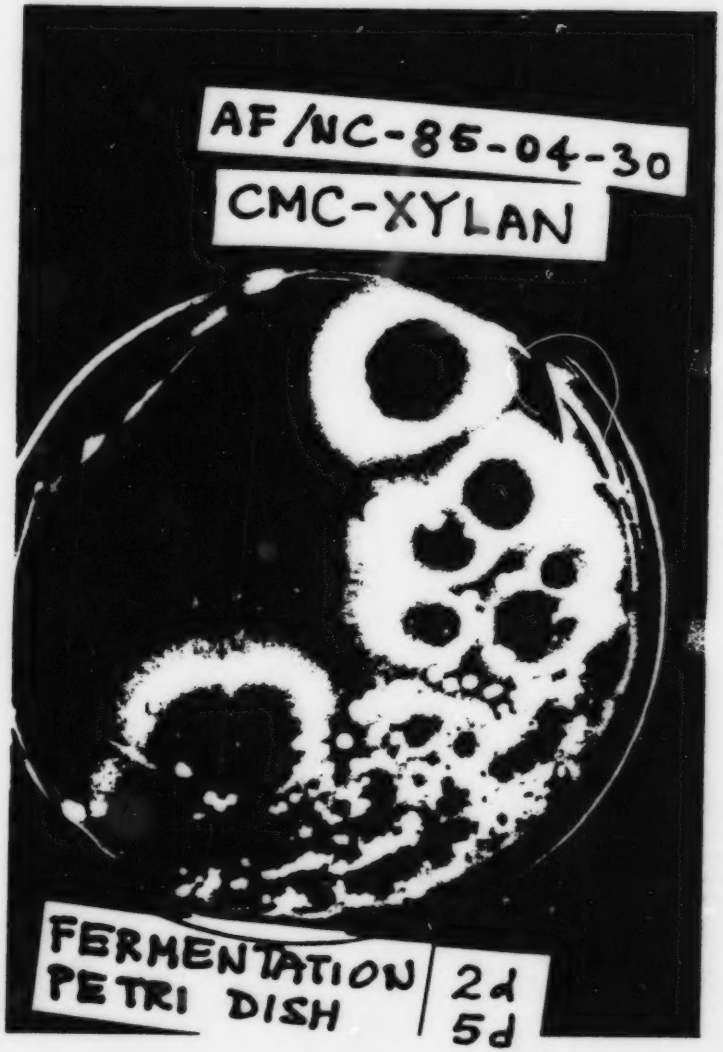


Fig. 3.35 - Microflora in bioreactors on the second day.

Fig. 3.36 - Microflora in bioreactors on the third day.

Fig. 3.37 - Microflora in bioreactors on the fourth day.

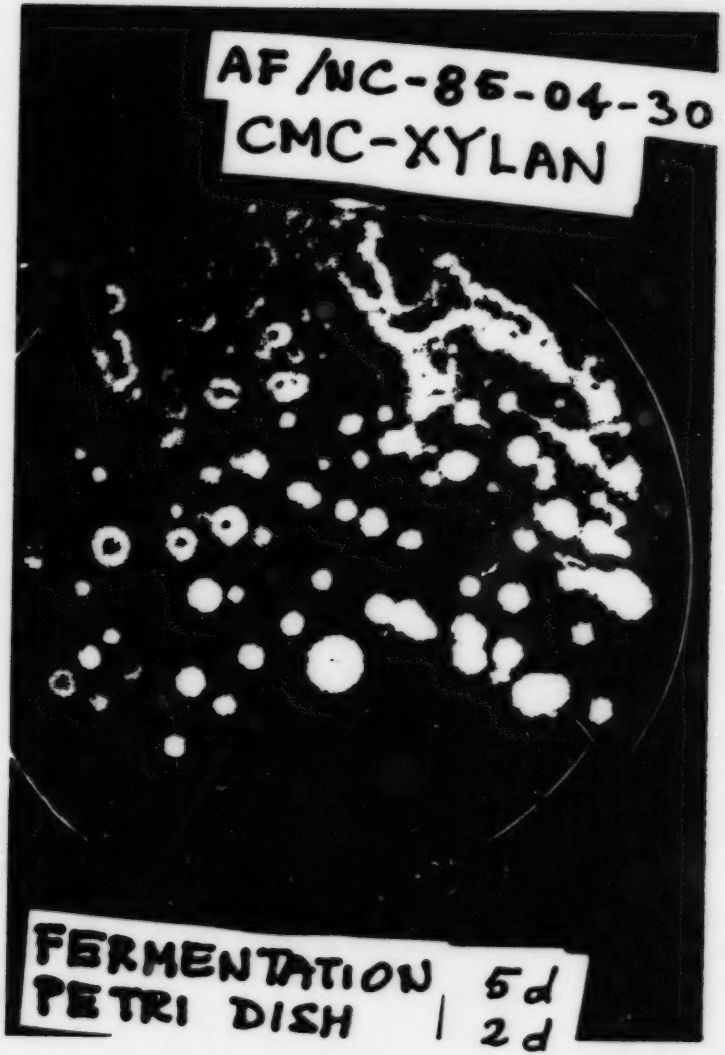


Fig. 3.38 - Microflora in bioreactors on the fifth day.

cellulolytic enzymes. This is because a relatively high inoculum concentration (2 - 3 g/ℓ) had been used to seed the bioreactors, and consequently, the acidophilic fungus population was not depleted by the yeast contaminant overgrown in the bioreactors.

During several attempts at continuous fermentation, the yeast contamination problem became more severe. Yeast contaminant grew in 150 ℓ reservoirs containing fresh-feed medium at pH 2,0. In addition, several high dilution rates applied to the bioreactors during these experiments, favored the retaining of the yeast contaminant (because of its relatively fast growing rate) and washed-out the acidophilic fungus mycelium (because of its very slow growing rate).

The major contaminant occurring in an acidic medium of pH 2,0 was only one unidentified strain of yeast. This contaminant yeast multiplied by budding and could not grow on pure cellulosics or lignocellulosics. Another contaminant occasionnally found in the culture medium at pH 2,0 was Aureobasidium pullulans. This yeast-like fungus was able to grow under such acidic pH conditions (Lacroix et al. 1985). Another contaminant rarely and insignificantly found in an acidic medium of pH 2,0 was Aspergillus sp.

3.4.2 Identity of the acidophilic fungus

Because of the contamination problem in continuous fermentation experiments and because of the unsuccessful results from the induction and mutation experiments, our efforts concentrated on the search for an explanation during the last phase of the project, as indicated by Task (h) in Figure 2.4.

In the first place, a knowledge of the identity of the acidophilic fungus strain NC-11 seemed essential. Partial identification of this

fungus done in our laboratory according to the procedure established by Raper and Thom 1968 revealed that the acidophilic fungus was of the genus Penicillium (figure 3.39). Further identification of the species name was confined to the Mycological Services at the American Type Culture Collection (U.S.A.). It was found that our acidophilic fungus NC-11 is a known species under the scientific name of Penicillium ochro-chloron. The complete report on the identification by the American Type Culture Collection is found in Appendix C.

Penicillium ochro-chloron has been known to be among the fungi tolerant of high concentrations of heavy metals and acidic environments. It has been isolated from metal-contaminated lake and stream water, and from industrial acidic copper-plating solution. In laboratory, P. ochro- chloron grew at any pH from 2,0 to 8,0 (Stokes and Lindsay 1979) and can grow in a medium saturated with copper sulfate, e.g. 40% $CuSO_4.5H_2O$ solution (Ikamoto and Fuwa 1974). A detailed study of the tolerance and uptake of other metals by P. ochro-chloron has been done by Okamoto et al. 1977. These authors found the following toxicity limits, e.g. the concentrations at which spore germination of the fungus was completely inhibited, of P. ochro-chloron for different metals: Cu (saturated; >100 g/ℓ); Zn (saturated; >100 g/ℓ); Mn (saturated; >100 g/ℓ); Fe (20 g/ℓ); Cd (20 g/ℓ); Pb (20 g/ℓ); Cr (3g/ℓ); Co (2 g/ℓ); Tl (1 g/ℓ); Ni (0,2 g/ℓ), and Hg (0,01 g/ℓ). This explained why our acidophilic fungus had been isolated from a sulfochromic solution (sodium dichromate-sulfuric acid solution) which has been used for soaking glassware in a laboratory (Boa and LeDuy 1982). Because of the above properties, Penicillium ochro-chloron has a potential application for the removal and recovery of metals from wastewaters.

The cellulolytic activity of P. ochro-chloron has been reported in the literature by Reese et al. 1962. When grown in β-(1→6)-glucan as substrate, P. ochro-chloron QM 477 produced 16,8 U of β-(1→6)-glucanase per mℓ of medium, which was almost nine times that produced by Tricho-

Fig. 3.39 - Conidia and conidiospores of the acidophilic fungus (1000 X).

derma viride QM 6a (Reese et al. 1962). The optimum β-(1→6)-glucanase activity of P. ochro-chloron was at pH 4 and at 60 °C, as reported by these authors. Such optimal conditions are in concordance with those found for our acidophilic fungus strain NC-11 (Cauchon and LeDuy 1985).

3.4.3 Suggested solutions for the contamination problem

(i) It is clear that the extreme conditions of pH 2,0 of the culture medium alone cannot warrant axenic fermentation with acidophilic fungus under nonsterile and nonaseptic conditions, especially for large-scale operations. It was found that an unidentified contaminant yeast strain and also the yeast-like fungus Aureobasidium pullulans could grow in a culture media at pH 2,0.

Because P. ochro-chloron can grow in a medium saturated with Cu^{++}, Zn^{++} or Mn^{++}, it would be of interest to find out the toxicity level of such metals on the yeast contaminant (and on Aureobasidium pullulans). If this toxicity level is below the inhibition level for growth and for cellulolytic enzyme production of acidophilic fungus, then a potential solution for the contamination problem would be the fermentation of metal(s). The practical problem of this solution is to find economical methods for reusing metals in fermentation plants and for removing of metals(s) from the enzyme product.

(ii) The yeast contaminant did not significantly affect the fermentative production of cellulolytic enzymes under nonsterile and nonaseptic conditions when the acidophilic fungus-yeast contaminant ratio was reasonably high. Such was the case in batch fermentation experiments and biodisc fermentation experiments. Therefore, a fermentation method with a high density of acidophilic fungus (such as fermentation using immobilized cells) will be essential as a solution to the contamination problem.

(iii) The contaminant grew on fermentable sugars resulting from the hydrolytic action of cellulolytic enzymes produced by acidophilic fungus during fermentation of the cellulosic substrates in the culture medium. An economical method for continuous removal of fermentable sugars will be helpful for inhibitting the development of the contaminants.

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

4.1 CONCLUSIONS

(1) It was possible to use an acidophilic cellulolytic fungus strain NC-11 (deposited as patent Culture ATCC-20677) and recently identified as Penicillium ochro-chloron) for the fermentative production of extracellular cellulolytic enzymes, under nonsterile and nonaseptic conditions. The culture medium consisted of a carbohydrate substrate in a simple basal salt solution of K_2HPO_4 0,1%; $(NH_4)_2SO_4$ 0,1%; $NaNo_3$ 0,1% and $MgSO_4.7H_2O$ 0,016% at pH 2,0 (adjusted with H_2SO_4). Fermentation was conducted at temperatures of 27-30 °C and with uncontrolled of pH.

(2) The maximum cellulolytic activities of the culture filtrates obtained from batch fermentatin in 10 ℓ (working volume) bioreactor were: 1 IU of xylanase, 0,15 IU of CMCase and 0,02 IU of FPase in CMC-xylan medium; 0,05 IU of xylanase, 0,015 IU of CMCase and 0,02 IU of FPase in newspaper medium.

(3) In CMC-xylan medium, both xylanase and CMCase production were growth associated according to

$$\frac{d[\text{xylanase activity}]}{dt} = 88,84 \frac{d[\text{ATP concentration}]}{dt}$$

and

$$\frac{d[\text{CMCase activity}]}{dt} = 9,63 \frac{d[\text{ATP concentration}]}{dt}$$

In newspaper medium, only CMCase production was associated with growth according to

$$\frac{d[\text{CMCase activity}]}{dt} = 14{,}24 \frac{d[\text{ATP concentration}]}{dt}$$

(4) In Erlenmeyer batch fermentation, no extracellular cellulolytic enzymes were produced with any of the following carbohydrate substrates: glucose, sucrose, cellobiose, maltose, fructose, galactose, lactose, xylose and starch. None of the following cellulosic and lignocellulosic substrates significantly produced FPase activity (less than 0,025 IU): Avicel cellulose, cellulose powder, α-cellulose, Na-CMC, xylan, newspaper, newsprint, sawdust from softwood, cardboard carton and a mixture of CMC-xylan.

(5) Under nonsterile and nonaseptic fermentation conditions, an unidentified yeast contaminant was developed in the bioreactor. However, yeast contamination did not affect cellulolytic enzyme production in the presence of high concentrations of acidophilic fungus mycelium in the bioreactor.

(6) Continuous fermentation in the bioreactor using free cells of acidophilic fungus was not possible: the yeast contaminant overgrew owing to its faster growth rate and the acidophilic fungus mycelium was washed out because of its slower growth rate.

(7) Cellobiose, Na-CMC, xylan, newspaper and sawdust from softwood had no inducing power over the synthesis and excretion of cellulolytic enzymes of acidophilic fungus.

(8) Several mutation tests on acidophilic fungus spores with UV radiation, X-ray radiation, ethyl methanesulfonate, N-methyl-N'-nitro-N-nitrosoguanidine and nitrous acid did not yet result in obtaining a mutant of acidophilic fungus with higher FPase activity.

(9) An improved modified Lowry method for the determination of low protein concentration in the culture filtrate was developed. All steps of the analytical procedure were done in one 1,5 mℓ Eppendorf Micro Test Tube. This improved procedure has the advantage of no sample (protein) loss owing to transfers and less handling of glassware.

(10) A non-linear effect of dilution (or enzyme concentration) on the cellulolytic enzyme assays (xylanase, CMCase, FPase) existed. Consequently, when samples contained either too much or too little enzyme (which should be diluted or concentrated prior to the assay procedure), a conventional multiplicative correction factor (for diluted samples) or divisional correction factor (for concentrated samples), successfully used for other enzyme assays, could not be applied for cellulolytic enzymes.

(11) Two common dyes used for screening tests of cellulolytic microorganisms, Congo Red and Remazol Brilliant Blue R, cannot be used for rapid screening of the acidophilic fungus mutants. Under an acidic condition of pH 2,0, these two dyes were precipitated.

4.2 RECOMMENDATIONS FOR FUTURE WORK

(1) Congo Red and Remazol Brilliant Blue cannot be used in a rapid screening test for high FPase-producing mutants of acidophilic fungus owing to the fact that they precipitate under acidic conditions. A new screening method, either based on another dye or based on a completely new principle, has to be developed.

(2) When a rapid screening test in (1) is available, several thousands of mutation tests have to be performed in order to obtain conclusive results.

(3) The extreme condition of pH 2,0 of the culture medium alone cannot warrant an axenic fermentation with acidophilic fungus under nonsterile and nonaseptic conditions, especially for large-scale operations. Another supplementary extreme condition, together with the pH 2,0 has to be found. This supplementary extreme condition should not affect the production and excretion of cellulolytic enzymes by the acidophilic fungus, but will inhibit the development of the yeast contaminant (and also Aureobasidium pullulans, a potential contaminant). Such supplementary extreme condition could be a high concentration of metal ions, organic solvents, etc.

(4) The contaminant grew on fermentable sugars resulting from the hydrolytic actions of cellulolytic enzymes produced by acidophilic fungus during fermentation of the cellulosic substrates in the culture medium. An economical method for continuous removal of fermentable sugars produced (for inhibiting the development of the contaminants) will be an alternative solution to the contamination problem.

REFERENCES

Anonymous (1982): University of Arkansas: The Cellulose-to-Ethanol Process, in Alcohol Fuels Program Technical Review, Solar Energy Research Institute, pp. 57-74.

Atkinson, B. and Mavituna, F. (1983): Biochemical Engineering and Biotechnology Handbook, The Nature Press.

Avalos, J., Casadesus, J., and Cerda-Olmedo, E. (1985): Gribberella fujikuroi: Mutants Obtained with UV Radiation and N-Methyl-N'-Nitro-N-Nitrosoguanidine, Appl. Environ. Microbiol., 49: 187-191.

Bel'amy, W.D. and Chakrabarty, A.M. (1974): Solube Cellulase Enzyme Production, U.S. Patent No. 3,812,013.

Boa, J.M. and LeDuy, A. (1982): Acidophilic Fungus SCP from Peat Hydrolyzate, Can. J. Chem. Eng., 60: 532-537.

Carlton, B.C. and Brown, B.J. (1981): Gene Mutation, in "Manual of Methods for General Bacteriology", American Society for Microbiology, Washington, DC, U.S.A., pp. 222-242.

Chahal, D.S. (1982): Enzymatic Hydrolysis of Cellulose: State-of-the-Art, National Research Council Canada.

Cauchon, N. (1984): Isolement et caractérisation d'un fungus acidiphile cellulolytique: production des enzymes et évaluation partielle des propriétés biochimiques, M. Sc. Thesis, Université Laval.

Cauchon, N. and LeDuy, A. (1984): Effect of Dilution on Carboxymethylcellulase and Xylanase Assays, Biotechnol. Bioeng., 26: 988-991.

Cauchon, N. and LeDuy, A. (1985): Novel Process for the Production of Cellulolytic Enzymes, Biotechnol. Bioeng., 27: 456-462.

Daigneault-Sylvestre, N., and Kluepfel, D. (1979): Method for Rapid Screening of Cellulolytic Streptomycetes and Their Mutants, Can. J. Microbiol. 25: 858-860.

Emert, G.H., Katzen, R., Fredrickson, R.E., and Kaupisch, K.F. (1980): Economic Update of the Gulf Cellulose Alcohol Process, Chem. Eng. Progress, 76(9): 47-52.

Eriksson, K.-E. and Johnsrud, S.C. (1983): Mutants of the White-Rot Fungus Sporotrichum pulverulentum with Increased Cellulase and β-D-Glucosidase Production, Enzyme Microb. Technol., 5: 425-429.

Gallo, B.J., Andreotti, R., Roche, C., Ryu, D., and Mandels, M. (1978): Cellulase Production by a New Mutant Strain of Trichoderma reesei MCG 77, Biotechnol. Bioeng. Symp., 8,: 89-101.

Ghosh, V.K., Ghose, T.K., and Gopalkrishnan K.S. (1982): Improvement of T. reesei Strain Through Mutation and Selective Screening Techniques, Biotechnol. Bioeng., 24: 241-243.

Hagerdal, B., Ferchak, J.D., and Pye, E.K. (1980): Saccharification of Cellulose by the Cellulolytic Enzyme System of Thermomonospora sp. I. Stability of Cellulolytic Activities with Respect to Time, Temperature, and pH, Biotechnol. Bioeng., 22: 1515-1526.

Horikoshi, K., Nakao, M., Kurono, Y., and Sashihara, N. (1984): Cellulases of an Alkalophilic Bacillus Strain Isolated from Soil, Can. J. Microbiol., 30: 774-779.

Khan, A.W., Wall, D., and Van den Berg, L. (1981): Fermentation Conversion of Cellulose to Acetic Acid and Cellulolytic Enzyme Production by a Bacterial Mixed Culture Obtained for Sewage Sludge, Appl. Environ. Microbiol., 41: 1214-1218.

Labudova, I., Farkas, V., Bauer, S., Kolarova, N., and Branyik, A. (1981): Characterization of Cellulolytic Enzyme Complexes Obtained from Mutants of Trichoderma reesei with Enhanced Cellulase Production, Eur. J. Appl. Microbiol. Biotechnol., 12: 16-21.

Lacroix, C., LeDuy, A., Noël, G., and Choplin, L. (1985): Effect of pH on the Batch Fermentation of Pullulan from Sucrose Medium, Biotechnol. Bioeng., 27: 202-207.

LeDuy, A. and Côté, H. (1985): Biosynthesis of Carboxymethylcellulase and Xylanase by Acidophilic Fungus Cells Immobilized on Biodiscs, 85th Annual Meeting Amer. Soc. Microbiol., Las Vegas, Nevada, U.S.A., March 3-7, 1985.

Leisola, M., and Linko, M. (1976): Determination of the Solubilizing Activity of a Cellulase Complex with Dyed Substrates, Anal. Biochem. 70: 592-599.

Leisola, M., and Linko, M. (1977): Use of Dyed Avicel Cellulose to Determine the Solubilizing Activity of a Cellulase Complex, Finn. Chem. Lett.: 172-176.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951): Protein Measurement with the Folin Phenol Reagent, J. Biol. Chem., 193: 265-275.

Luedeking, R. and Piret, E.L. (1959): A Kinetic Study of the Lactic Acid Fermentation, J. Biochem. Microbiol. Technol. Eng., 1: 393-431.

Mandels, M., Andreotti, R., and Roche, C. (1976): Measurement of Saccharifying Cellulase, Biotechnol. Bioeng. Symp., 6: 21-33.

Mandels, M., Weber, J. and Parizek, R. (1971): Enhanced Cellulase Production by a Mutant of Trichoderma viride, Appl. Microbiol., 21: 152-154.

Miller, G.L. (1959): Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar, Anal. Chem. 31: 426-428.

Mishra, S., Gopalkrishnan, K.S., and Ghose, T.K. (1982): A Constitutively Cellulase-Producing Mutant of Trichoderma reesei, Biotechnol. Bioeng., 24: 251-254.

Montenecourt, B.S., Kelleher, T.J., and Eveleigh, D.E. (1980): Biochemical Nature of Cellulases from Mutants of Trichoderma reesei, Biotechnol. Bioeng. Symp. No. 10, pp. 15-26.

Moreira, A.R., Phillips, J.A., and Humphrey, A.E. (1981): Production of Cellulases by Thermomonospora sp., Biotechnol. Bioeng., 23: 1339-1347.

Nevalainen, K.M.H. and Palva, E.T. (1978): Production of Extracellular Enzymes in Mutants Isolated from Trichoderma viride Unable to Hydrolyse Cellulose, Appl. Environ. Microbiol., 35: 11-16.

Ng, T.K., Weimer, P.J., and Zeikus, J.G. (1977): Cellulolytic and Physiological Properties of Clostridium thermocellum, Arch. Microbiol., 114: 1-7.

Ng, T.K., and Zeikus, J.G. (1980): A Continuous Spectrophotometric Assay for the Determination of Cellulase Solubilizing Activity, Anal. Biochem., 103: 42-50.

Nummi, M., Fox, P.C., Niku-Paavola, M.-L. and Enari, T.-M. (1981): Nephelometric and Turbidometric Assays of Cellulase Activity, Anal. Biochem., 116: 133-136.

Okamoto, K., and Fuwa, K., (1974): Copper Tolerance of a New Strain of Penicillium ochro-chloron, Agr. Biol. Chem., 38: 1405-1406.

Okamoto, K., Suzuki, M., Fukami, M., Toda, S., and Fuwa, K., (1977): Uptake of Heavy Metals by a Copper-Tolerant Fungus, Penicillium ochro-chloron, Agric. Biol. Chem., 41: 17-22.

Poincelot, R.P. and Day, P.R. (1972): Simple Dye Release Assay for Determining Cellulolytic Activity of Fungi, Appl. Microbiol., 23: 875-879.

Raper, K.B. and Thom, C. (1968): A Manual of the Penicillia, Hafner Publishing Co., New York, 1968.

Reese, E.T. and Mandels, M. (1980): Stability of the Cellulase of Trichoderma reesei under Use Conditions, Biotechnol. Bioeng., 22: 323-335.

Reese, E.T. Parrish, F.W., and Mandels, M. (1962): β-D-1,6-Glucanases in Fungi, Can. J. Microbiol., 8: 327-334.

Rho, D., Desrochers, M., Jurasek, L, Driguez, H., and Defaye, J. (1982): Induction of Cellulase in Schizophyllum commune: Thiocellobiose as a New Inducer, J. Bacteriol., 149: 47-53.

Ryu, D., Andreotti, R., Mandels, M., Gallo, B., and Reese, E.T. (1979): Studies on Quantitative Physiology of Trichoderma reesei with Two-Stage Continuous Culture for Cellulase Production, Biotechnol. Bioeng., 21: 1867-1903.

Shoemaker, S., Watt, K., Tsitovsky, G., and Cox, R. (1983a): Characterization and Properties of Cellulases Purified from Trichoderma reesei Strain L27, Bio/Technology, 1: 687-690.

Shoemaker, S., Schweickart, V., Ladner, M., Gelfand, D., Kwok, S., Myambo, K., and Innis, M. (1983b): Molecular Cloning of Exo-cellobiohydrolase I Derived from Trichoderma reesei Strain L27, Bio/Technology, 1: 691-696.

Skinner, W.A. and Tokuyama, F. (1978): Production of Cellulase by a Thermophilic Thielavia terrestris, U.S. Patent No. 4,081,328.

Smith, P.W. (1984): Towards the Implementation of the Iotech Process, Proceedings 6th International Symp. on Alcohol Fuels Technol., 3: 503-506.

Smith, R.E. (1977): Rapid Tube Test for Detecting Fungal Cellulase Production, App. Env. Microbiol., 33: 980-981.

Sternberg, D. and Mandels, G.R. (1979): Induction of Cellulolytic Enzymes in Trichoderma reesei by Sophorose, J. Bacteriol., 139: 761-769.

Sternberg, D., and Mandels, G.R. (1980): Regulation of the Cellulolytic System in Trichoderma reesei by Sophorose: Induction of Cellulase and Repression of β-Glucosidase, J. Bacteriol., 144: 1197-1199.

Stokes, P.M. and Lindsay, J.E. (1979): Copper Tolerance and Accumulation in Penicillium ochro-chloron Isolated from Copper-Plating Solution, Mycologia, 71: 796-806.

Stone, J.E. (1982): Analysis of Ethanol Production Potential from Cellulosic Feedstocks, Energy Mines Resources Canada.

Tan, L.U.L., Chan, M.K.-H., and Saddler, J. (1984): A Modification of the Lowry Method for Detecting Protein in Media Containing Lignocellulosic Substrates, Biotechnol. Letters, 6: 199-204.

Tangnu, S.K., Blanch, H.W., and Wilke, C.R. (1981): Enhanced Production of Cellulase, Hemicellulase, and β-Glucosidase by Trichoderma reesei (Rut-C30), Biotechnol. Bioeng., 23: 1837-1849.

Teather, R.M. and Wood, P.J. (1982): Use of Congo Red-Polysaccharide Interactions in Enumeration and Characterization of Cellulolytic Bacteria from th Bovine Rumen, Appl. Environ., Microbiol., 43: 777-780.

Teeri, T., Salovuori, I., Knowles J. (1983): The Molecular Cloning of the Major Cellulase Gene from Trichoderma reesei, Bio/Technology, 1: 696-699.

Weiss, R.M. and Ollis, D.F. (1980): Extracellular Microbial Polysaccharides. I. Substrate, Biomass and Product Kinetic Equations for Batch Xanthan Gum Fermentation, Biotechnol. Bioeng., 22: 859-873.

APPENDIX A

ANALYTICAL PROCEDURES

A.1 ATP CONCENTRATION

A.1.1 Reagents

(a) Tris buffer (0,02 M): Dissolve 2,4228 g of Tris (hydroxymethyl)aminomethane (Ultrol, Calbiochem No. 648315) in 1 ℓ distiled water. Adjust to pH 7,75 with NaOH 10 N solution. Sterilize the buffer solution in an autoclave, at 121 °C and 15 psig for 20 minutes.

(b) Luciferin-luciferase Enzyme solution: Add 5 mℓ of sterile distilled water in each firefly extract capsule FLE-50 (Sigma). This solution has to be freshly prepared. Each capsule FLE-50 can assay about 20 samples. Individual standard calibration curve has to be prepared with each FLE-50 capsule. When more than 20 samples are to be assayed (not exceeded 80 for each run), one standard calibration curve can be obtained from a mixture of luciferin-luciferase coming from several FLE capsules. All manipulation has to be under sterile and aseptic conditions.

(c) ATP solution: Add 10 mℓ of (sterile) Tris buffer to each ATP capsule (Sigma, No. A-3127, FFATP) and mix well. This solution has to be fresly prepared.

A.1.2 Extraction of ATP

ATP of the sample (culture medium containing microbial cells) has to be extracted immediately after sampling. Transfer 1 mℓ of homogeneous sample in a 50 mℓ volumetric flask containing 40 mℓ of boiling Tris buffer (has previously been placed in a water bath at 100 °C for 10 minutes). Incubate at 100 °C for 10 minutes, then cool to room temperature in an ice bath. Fill the volumetric flask to 50 mℓ level with Tris buffer, mix well and transfer to sampling vial for immediate storage at -20 °C.

A.1.3 Preparation of the standard calibration curve

Step:

(a) Turn POWER on. OVERLOAD will flash, Depress RESET. Allow the instrument to warm-up and stabilize for at least 15 minutes.

(b) Prepare ATP standards: Sterile distilled water must be used in all sample, enzyme and ATP preparations. Mark the freshly prepared ATP solution (A.1.1c) vial "A". This vial may be preserved by freezing for future re-use. Mark six scintillation vials B, C, D, E, F, G. Dilute ATP standard according to the following ratio [Caution: when doing serial dilutions, never use the same pipette tip twice]:

Vial	Tris buffer (mℓ)	Add to	Standard concentration (mg ATP/mℓ)
B	19,8	0,2 mℓ solution A	1
C	9,5	0,5 mℓ solution B	0,05
D	8,0	2,0 mℓ solution C	0,1
E	9,0	1,0 mℓ solution C	0,005
F	9,0	1,0 mℓ solution B	0,1
G	1,0	1,0 mℓ solution C	0,025

The solutions C to G will normally be used to develop a standard curve (shown in Figure A.1.1). Cap the standard solutions to prevent evaporation and contamination.

(c) Zero the instrument with the SENSITIVITY dial at a preselected setting of 7 to 8,5. The sensitivity of the instrument is increased logarithmically as the setting number increases. Therefore, the larger the concentration of the ATP to be assayed, the lower setting of the sensitivity. A high setting on high concentrations will cause a very fast count and possibly an overload.

(d) Dispense 0,2 mℓ of prepared Enzyme (A.1.1b) into seven scintillation vials (only six are required for standards and blank, however, seven are recommended in case of a pipetting error or re-checking to establish a data point).

(e) Set instrument for "INTEG", and "6 SEC".

(f) Insert one vial containing only the Enzyme into sample chamber, cover with the cap, pull the shutter, and depress ASSAY switch. The number is the background activity of the enzyme. This reading will run anywhere from less than 100 to possibly 1 000 with a crude enzyme. The background number will degrade over a period of time - this is normal and due to the reaction of trace amounts of cellular ATP left over from the firefly insect's body. Record data as shown in Table A.1.1 [Note: The 6 second reading automatically multiplies by 10 to give an equivalent reading to 60 seconds].

Blank determination

(g) Depress RESET and set time to "60 SEC" mode. Push shutter in, and remove vial with Enzyme from chamber. Pipet 0,5 mℓ of the Tris buffer (which is considered as blank). Simultaneously, while injecting the Tris buffer into the sample vial of Enzyme, step on the footswitch (this is a step/inject motion). The footswitch starts the assay time. Within the next 15 seconds, swirl the mixture, place the vial into the sample chamber, cap, and pull the shutter. After the delay, the instrument will assay for 60 seconds. Record the number as shown in Table A.1.1. Discard the vial. This reading is the reagent blank for zero concentration of ATP. This number will be deducted from all future sample readings.

Standard and sample determination

(h) Depress RESET and set time to "6 SEC" mode. Measure background activity as described in Step (f). Record the data under 6 Second heading for ATP Standard E as shown in Table A.1.1.

(i) Depress RESET and set time to "60 SEC" mode. Pipet 0,5 mℓ of ATP Standard "E". Use the same procedure (step/inject) for injecting and starting assay as described in Step (g). Record the data under 60 Second heading for ATP Standard "E" as shown in Table A.1.1.

(j) Repeat the same procedures (h) and (i) for measuring background of enzyme and ATP Standards D, C, F and G.

(k) The data must be corrected by deducting the blank reading as shown in Table A.1.1. The corrected data are plotted on 3 x 3 cycle log paper as shown in Figure A.1.1.

A.2 PROTEIN CONCENTRATION

A.2.1 Reagents

(a) Solution A: Dissolve 30 g of Na_2CO_3 in 1 ℓ distilled water.

(b) Solution B: Dissolve 20 g of $CuSO_4.5H_2O$ in 1 ℓ distiled water.

(c) Solution C: Dissolve 40 g of Na-tartrate. $2H_2O$ in 1 ℓ distilled water.

(d) Alkaline copper reagent: Mix solutions A, B and C in a 48:1:1 mixture. This reagent has to be freshly prepared.

(e) Phenol reagent: Folin-Ciocalteau reagent (BDH) in two volumes of distiled water.

(f) Standard solution of protein: Dissolve 0,5 g of Bovine Albumine (Sigma) in 1 ℓ distilled water. This mother solution is used to prepare various standard solutions from 5 to 50 mg/ℓ for the standard curve.

A.2.2 Preparation of the standard calibration curve

Follow the procedure described in paragraph 2.3.5 for standard calibration curve. Example of a standard calibration curve is shown in Figure A2.1 (and Table A2.1).

A.3 ENZYME ACTIVITIES

A.3.1 Reagents

(a) Acetate buffer at pH 3,0: Mix 8,66 mℓ of acetic acid (glacial) in 1 ℓ distilled water to obtain 0,15 M acetic acid solution. Add 0,15 M sodium acetate solution into 0,15 M acetic acid solution until the pH of the mixture reaches 3,0.

(b) CMC solution at 1%: Dissolve 10 g of Na-CMC (Sigma, low viscosity) in 1 ℓ acetate buffer at pH 3,0 (a).

(c) Xylan solution at 1%: Dissolve 10 g of xylan (Sigma, from larchwood) in 1 ℓ acetate buffer at pH 3,0 (a).

(d) Glucose buffer: Dissolve 0,13 g of glucose in 1 ℓ acetate buffer at pH 3,0 (a).

(f) Glucose standard solution: Dissolve 4 g of glucose in 1 ℓ distilled water. This mother solution will be used for the preparation of various standard solutions for the standard calibration curves for glucose.

(g) Dinitrosalycilic acid reagent: Dissolve the following chemicals in 2ℓ distilled water: NaOH 40 g; dinitrosalicylic acid 20 g; sodium sulfite 1 g; phenol 4 g. This reagent is stable for 2 to 3 weeks in the refrigerator.

(h) Filter paper strips: Filter paper Whatman No. 1 cut into 1 cm x 6 cm size.

A.3.2 Preparation of the standard calibration curves

(a) Standard curve for low concentrations of glucose (2 g/ℓ): Follow the procedure described in paragraph 2.3.6 and Table 2.2 (for the tubes SC). Example of the standard calibration curve is shown in Figure A3.1 (and Table A3.1).

(b) Standard calibration curve for high concentrations of glucose (4 g/ℓ): The same as above (a), except that 16 mℓ of distilled water are added to the tubes prior to reading in the spectrophotometer. Example of the standard calibration curve is shown in Figure A3.1 (and Table A3.1).

A.3.3 Enzyme assays

(a) CMCase and xylanase: Follow the procedure described in paragraph 2.3.6 and in Table 2.2.

(b) FPase: The same as above (a), except that 1,0 mℓ of glucose buffer is added to the tubes between the steps (3a) and (3b) indicated in Table 2.2.

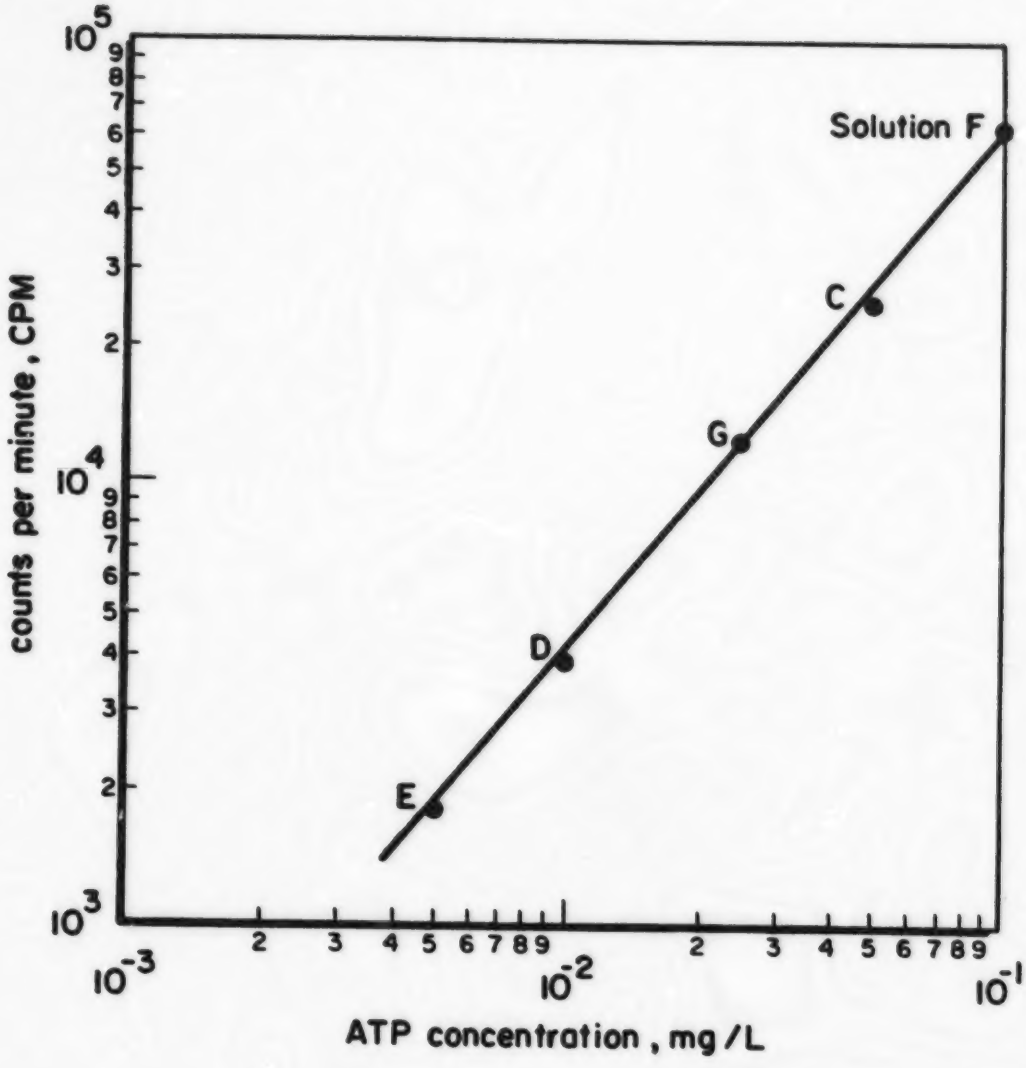


Fig. A1.1 - Standard calibration curve for ATP.

Table A1.1 - Standard calibration curve for ATP

Sample	Counts per minute (CPM)		
	6 sec Background	60 sec Reading	Corrected reading
Blank	3 307	2 992	0,904[a]
Standard E (5×10^{-3} mg/L)	3 366	4 849	1 809
Standard D (1×10^{-2} mg/L)	3 112	6 786	3 976
Standard C (5×10^{-2} mg/L)	3 389	28 751	25 691
Standard F (1×10^{-1} mg/L)	3 233	67 459	64 539
Standard G ($2,5 \times 10^{-2}$ mg/L)	3 309	15 222	12 234

(a) Correction factor.

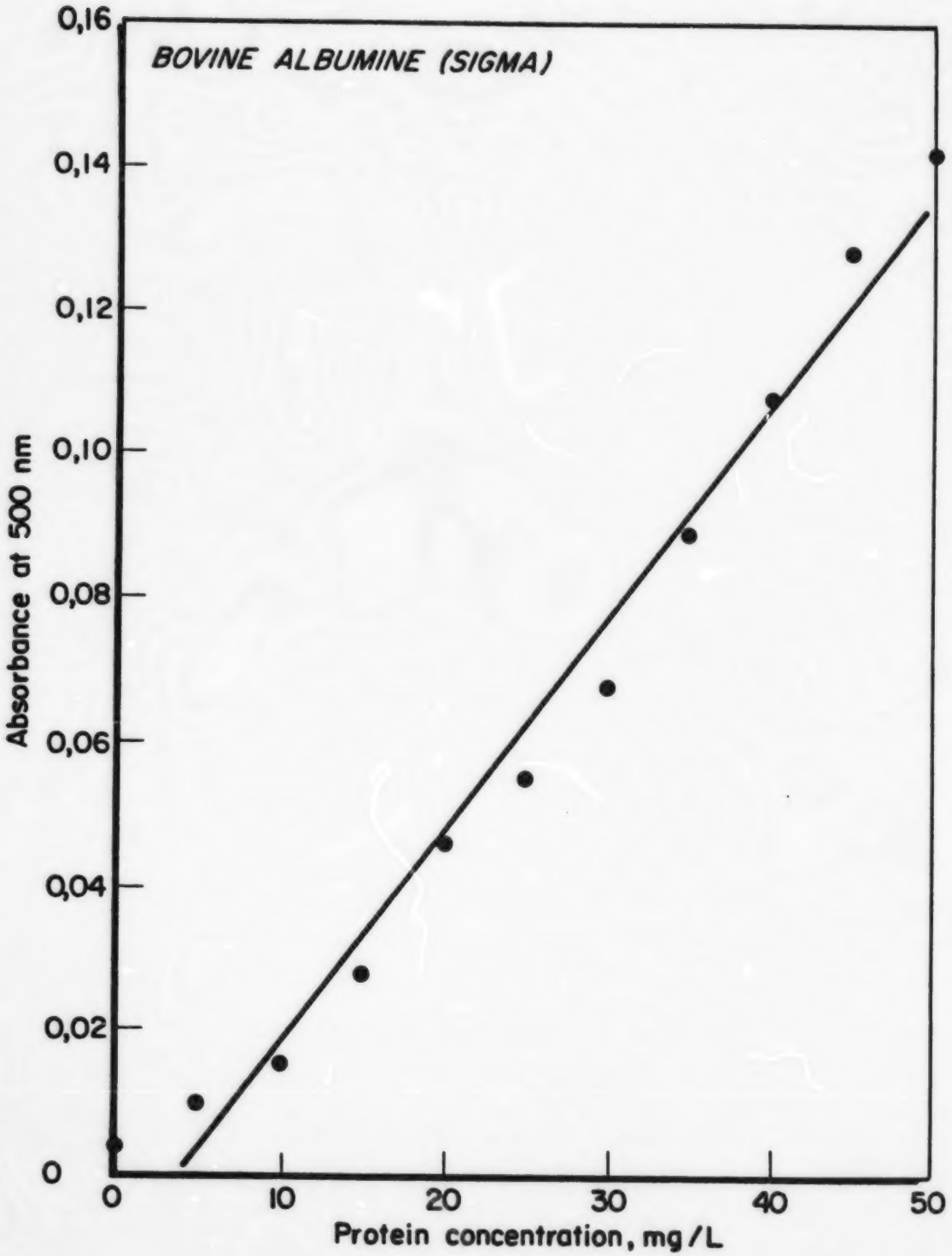


Fig. A2.1 - Standard calibration curve for protein.

Table A2.1 - Standard calibration curve for protein.

Protein concentration[a] (mg/L)	Absorbance at 500 nm											
	1	2	3	4	5	6	7	8	9	10	$\bar{x}$	s
0	0,000	0,001	0,000	0,000	0,000	0,001	0,000	0,000	0,000	0,002	0,0004	0,0006
5	0,013	0,012	0,001	0,001	0,010	0,018	0,010	0,014	-	-	0,0099	0,0056
10	0,020	0,012	0,006	0,008	0,015	0,016	0,018	0,021	0,018	0,022	0,0156	0,0051
15	0,025	0,034	0,025	0,030	0,027	0,029	0,029	0,027	-	-	0,0282	0,0075
20	0,031	0,048	0,048	0,041	0,050	0,056	0,050	0,056	0,040	0,045	0,0465	0,0072
25	0,048	0,045	0,048	0,047	0,065	0,062	0,062	0,066	-	-	0,0554	0,0085
30	0,074	0,074	0,057	0,060	0,073	0,072	0,074	0,072	0,073	0,058	0,0683	0,0066
35	0,077	0,081	0,088	0,085	0,085	0,095	0,101	0,090	-	-	0,0840	0,0071
40	0,114	0,116	0,089	0,095	0,122	0,104	0,123	0,111	0,108	0,106	0,1088	0,0104
45	0,130	0,132	0,115	0,112	0,132	0,141	0,138	0,130	-	-	0,1287	0,0095
50	0,158	0,150	0,140	0,142	0,155	0,155	0,140	0,155	0,126	0,122	0,1428	0,0115

(a) Bovine albumine (Sigma).

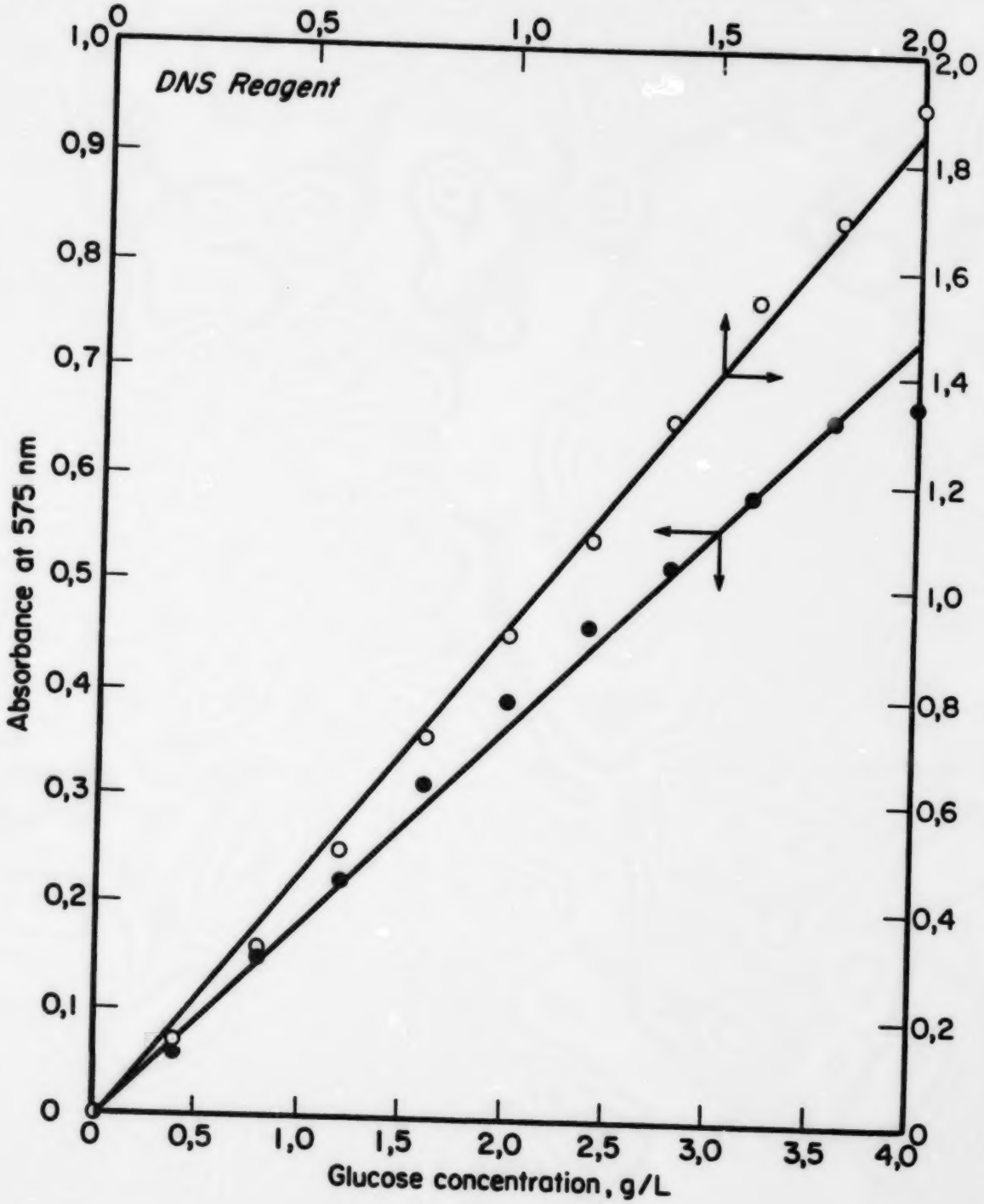


Fig. A3.1 - Standard calibration curves for glucose.

Table A3.1 - Standard calibration curves for glucose.

Low concentration curve				High concentration curve			
Glucose concentration (g/L)	Absorbance at 575 nm			Glucose concentration (g/L)	Absorbance at 575 nm		
	1	2	$\bar{x}$		1	2	$\bar{x}$
0,0	0,001	0,005	0,000	0,0	0,000	0,004	0,000
0,2	0,138	0,140	0,139	0,4	0,056	0,059	0,058
0,4	0,304	0,318	0,311	0,8	0,142	0,157	0,150
0,6	0,501	0,507	0,504	1,2	0,222	0,219	0,221
0,8	0,712	0,719	0,716	1,6	0,332	0,294	0,313
1,0	0,903	0,912	0,908	2,0	0,396	0,387	0,392
1,2	1,086	1,093	1,090	2,4	0,456	0,466	0,461
1,4	1,312	1,315	1,314	2,8	0,516	0,517	0,517
1,6	1,543	1,539	1,541	3,2	0,574	0,597	0,586
1,8	1,692	1,699	1,696	3,6	0,663	0,653	0,658
2,0	1,896	1,924	1,910	4,0	0,692	0,650	0,671

APPENDIX B

TABULATED RESULTS

Table B.1 - Effect of TCA precipitation time on the standard calibration curve for protein

Protein concentration[a] (mg/L)	Absorbance at 500 nm		
	Precipitation time 15 min	Precipitation time 60 min	Precipitation time 85 min
0	b	b	b
10	0,004	0,039	0,050
20	0,059	0,083	0,153
30	0,142	0,189	0,196
40	0,223	0,295	0,295
50	0,268	0,409	0,412

(a) Bovine albumine (Sigma).

(b) Negative value.

Table B.2 - Effect of enzyme dilution on the measured enzyme activities.

Dilution[a]	CMCase				Xylanase				FPase			
	Run 1		Run 2		Run 1		Run 2		Run 1		Run 2	
	U	IU	U	IU	U	IU	U	IU	U	IU	U	IU
1	53,6	0,30	81,1	0,45	154,4	0,86	197,6	1,10	39,3	0,22	41,4	0,23
2	54,4	0,30	77,3	0,43	163,0	0,91	228,0	1,27	26,4	0,15	34,5	0,19
5	47,6	0,26	77,2	0,43	157,0	0,87	198,0	1,10	13,5	0,08	19,8	0,11
10	17,5	0,10	62,6	0,35	89,7	0,50	126,9	0,71	2,8	0,02	9,9	0,06
20	2,1	0,01	26,5	0,15	26,9	0,15	61,5	0,34	b	b	3,8	0,02
50	b	b	2,5	0,01	b	b	11,5	0,06	b	b	b	b
100	b	b	b	b	b	b	0,6	0,00	b	b	b	b

(a) When D = 1; there was no dilution; when D = x, the stock enzyme solution sample was diluted up to x times its initial volume. The stock enzyme solution contained 1% (w/v) of commercial *Trichoderma reesei* cellulase (Biocon, U.S.A.) in distilled water.

(b) Negative value.

Table B.3 - Enzymes production from various substrates : Xylanase.

Substrate	Day 0		Day 1		Day 7		Day 14	
	U	IU	U	IU	U	IU	U	IU
Avicel cellulose	5,5	0,03	8,9	0,05	31,8	0,18	14,5	0,08
Cellulose powder	7,0	0,04	5,1	0,03	2,5	0,01	1,7	0,01
α-Cellulose	6,6	0,04	9,2	0,05	2,1	0,01	2,1	0,01
Na-CMC	5,9	0,03	5,9	0,03	5,9	0,03	42,7	0,24
Xylan	11,5	0,06	14,1	0,08	119,4	0,66	39,3	0,22
Newspaper	11,1	0,06	6,6	0,04	8,1	0,05	8,5	0,05
Newsprint	18,6	0,10	15,3	0,09	18,3	0,10	10,4	0,06
Sawdust	19,4	0,11	15,3	0,09	17,1	0,10	13,0	0,07
Cardboard carton	17,5	0,10	11,1	0,06	14,5	0,08	4,7	0,03
CMC-Xylan	7,0	0,04	4,0	0,02	145,0	0,81	25,8	0,14

(a) Negative value.

Table B.4 - Enzyme production from various substrates : CMCase.

Substrate	Day 0		Day 1		Day 7		Day 14	
	U	IU	U	IU	U	IU	U	IU
Avicel cellulose	a	a	5,2	0,03	0,9	0,01	1,7	0,01
Cellulose powder	a	a	2,8	0,02	3,5	0,02	0,5	0,00
α-Cellulose	a	a	3,5	0,02	2,5	0,01	a	a
Na-CMC	a	a	3,0	0,02	1,1	0,01	5,8	0,03
Xylan	6,8	0,04	5,0	0,03	9,2	0,05	6,2	0,03
Newspaper	7,8	0,04	6,3	0,04	3,6	0,02	0,6	0,00
Newsprint	9,0	0,05	3,4	0,02	3,9	0,02	0,0	0,00
Sawdust	5,6	0,03	3,2	0,02	2,8	0,02	0,6	0,00
Cardboard carton	6,4	0,04	3,7	0,02	3,9	0,02	1,0	0,01
CMC-Xylan	2,1	0,01	4,7	0,03	17,3	0,10	3,5	0,02

(a) Negative value.

Table B.5 - Enzymes production from various substrates : FPase.

Substrate	Day 0		Day 1		Day 7		Day 14	
	U	IU	U	IU	U	IU	U	IU
Avicel cellulose	0,5	0,00	2,1	0,01	0,6	0,00	a	a
Cellulose powder	a	a	1,3	0,01	a	a	a	a
α-Cellulose	1,1	0,01	1,8	0,01	a	a	a	a
Na-CMC	0,4	0,00	1,6	0,01	a	a	a	a
Xylan	1,4	0,01	2,5	0,01	a	a	0,9	0,01
Newspaper	0,6	0,00	3,6	0,02	0,1	0,00	1,2	0,01
Newsprint	1,3	0,01	2,8	0,02	0,1	0,00	0,6	0,00
Sawdust	1,6	0,01	3,2	0,02	0,4	0,00	1,4	0,01
Cardboard carton	1,1	0,01	2,8	0,02	a	a	0,8	0,00
CMC-Xylan	0,7	0,00	2,5	0,01	0,3	0,00	0,6	0,00

(a) Negative value.

Table B.6 - Induction test with cellobiose.

Fermentation time (h)	Xylanase		CMCase		FPase	
	U	IU	U	IU	U	IU
0	5,3	0,03	3,2	0,02	0,1	0,00
1	6,1	0,03	3,2	0,02	a	a
2	6,4	0,04	2,9	0,02	0,1	0,00
3	6,8	0,04	5,5	0,03	a	a
4	4,3	0,02	4,9	0,03	a	a
5	8,7	0,05	4,8	0,03	0,3	0,00
6	4,8	0,03	4,0	0,02	a	a
7	3,9	0,02	0,6	0,00	a	a
8	5,4	0,03	4,8	0,03	1,3	0,01
9	5,6	0,03	3,2	0,02	0,1	0,00
10	4,5	0,03	3,0	0,02	a	a
11	7,0	0,04	1,2	0,01	0,3	0,00
12	5,3	0,03	1,1	0,01	a	a
24	2,9	0,02	1,9	0,01	a	a

(a) Negative value.

Table B.7 - Induction test with Na-CMC.

Fermentation time (h)	Xylanase		CMCase		FPase	
	U	IU	U	IU	U	IU
0	12,2	0,07	0,1	0,00	1,1	0,01
1	7,0	0,04	1,1	0,01	a	a
2	8,9	0,05	a	a	0,5	0,00
3	9,5	0,05	0,5	0,00	1,5	0,01
4	6,7	0,04	a	a	a	a
5	9,7	0,05	a	a	0,6	0,00
6	7,1	0,04	a	a	0,3	0,00
7	7,1	0,04	a	a	a	a
8	4,8	0,03	0,4	0,00	0,5	0,00
9	7,8	0,04	a	a	0,2	0,00
10	6,1	0,03	a	a	0,9	0,01
11	8,3	0,05	a	a	a	a
12	5,2	0,03	a	a	0,3	0,00
24	6,2	0,03	a	a	1,0	0,01

(a) Negative value.

Table B.8 - Induction test with xylan.

Fermentation time (h)	Xylanase		CMCase		FPase	
	U	IU	U	IU	U	IU
0	6,5	0,04	13,4	0,07	1,4	0,01
1	8,0	0,04	18,6	0,10	1,1	0,01
2	9,0	0,05	7,5	0,04	2,5	0,01
3	7,8	0,04	8,2	0,05	0,9	0,01
4	13,8	0,08	8,3	0,05	2,3	0,01
5	7,1	0,04	10,7	0,06	1,1	0,01
6	7,1	0,04	12,6	0,07	1,5	0,01
7	10,6	0,06	6,1	0,03	1,1	0,01
8	12,3	0,07	5,7	0,03	1,7	0,01
9	6,7	0,04	5,1	0,03	1,7	0,01
10	10,0	0,06	4,4	0,02	2,5	0,01
11	11,7	0,07	4,5	0,03	1,8	0,01
12	8,8	0,05	6,1	0,03	1,6	0,01
24	12,2	0,07	5,3	0,03	0,8	0,00

(a) Negative value.

Table B.9 - Induction test with newspaper.

Fermentation time (h)	Xylanase		CMCase		FPase	
	U	IU	U	IU	U	IU
0						
1						
0	a	a	0,4	0,00	0,1	0,00
1	a	a	0,9	0,01	a	a
2	0,9	0,01	0,2	0,00	a	a
3	1,5	0,01	0,4	0,00	a	a
4	0,7	0,00	0,7	0,00	a	a
5	a	a	0,7	0,00	0,1	0,00
6	a	a	a	a	0,3	0,00
7	a	a	2,7	0,02	a	a
8	0,7	0,00	0,1	0,00	0,1	0,00
9	1,7	0,01	1,0	0,01	0,7	0,00
10	a	a	a	a	a	a
11	a	a	6,7	0,04	0,1	0,00
12	a	a	0,7	0,00	a	a
24	0,1	0,00	1,5	0,01	0,0	0,00

(a) Negative value.

Table B.10 - Induction test with sawdust.

Fermentation time (h)	Xylanase		CMCase		FPase	
	U	IU	U	IU	U	IU
0	3,0	0,02	3,9	0,02	0,5	0,00
1	a	a	2,8	0,02	0,3	0,00
2	3,0	0,02	3,2	0,02	0,8	0,00
3	2,1	0,01	2,1	0,01	0,3	0,00
4	0,2	0,00	3,2	0,02	0,6	0,00
5	2,3	0,01	2,4	0,01	0,7	0,00
6	1,8	0,01	2,4	0,01	0,6	0,00
7	1,5	0,01	3,0	0,02	0,6	0,00
8	3,1	0,02	3,0	0,02	0,7	0,00
9	2,2	0,01	2,2	0,01	0,6	0,00
10	2,7	0,02	2,6	0,01	0,3	0,00
11	2,7	0,02	2,2	0,01	1,1	0,01
12	3,1	0,02	2,2	0,01	0,4	0,00
24	3,1	0,02	2,2	0,01	0,9	0,01

(a) Negative value.

Table B.11 - Batch fermentation in bioreactors : Variations of pH, temperature and DO concentration.

		Fermentation time (d)										
		0	1	2	3	4	5	6	7	8	9	10
Newsprint medium:												
Bioreactor 1A	pH	1,99	1,99	1,99	2,01	1,99	2,02	1,99	2,00	1,99	2,00	1,99
	T (°C)	27,5	28,0	28,0	27,4	27,0	27,5	28,0	28,0	27,5	27,5	27,6
	DO (mg/L)	10,6	10,8	9,9	10,1	8,7	9,1	8,7	9,6	8,5	8,7	8,5
Bioreactor 1B	pH	2,01	2,02	2,02	2,02	2,01	2,03	2,01	2,01	2,01	2,00	2,00
	T (°C)	27,0	27,5	27,8	27,2	27,0	27,0	28,0	28,0	27,4	27,6	27,5
	DO (mg/L)	10,5	10,2	10,2	8,8	8,7	8,9	9,7	10,0	8,5	8,6	8,3
CMC-xylan medium:												
Bioreactor 2A	pH	2,01	2,00	2,01	1,99	2,01	2,01	2,01	2,01	1,99	1,99	2,00
	T (°C)	27,2	28,0	27,5	27,4	26,8	27,0	29,0	27,0	27,4	27,5	27,5
	DO (mg/L)	10,4	11,4	9,6	6,4	7,5	8,0	7,8	9,3	8,3	8,5	8,2
Bioreactor 2B	pH	2,00	2,01	1,99	1,99	2,00	2,01	1,99	2,00	2,00	2,01	2,00
	T (°C)	26,5	28,2	28,0	27,0	26,5	27,2	28,0	27,5	27,6	27,5	27,6
	DO (mg/L)	10,6	9,6	7,2	7,4	8,2	7,6	8,0	9,5	8,4	8,6	8,6

Table B.12 - Batch fermentation in bioreactors : Variation of ATP concentration.

Fermentation time (h)	ATP concentration (mg/L)			
	Newsprint medium		CMC-xylan medium	
	Bioreactor 1A	Bioreactor 1B	Bioreactor 2A	Bioreactor 2B
0	-	0,00	0,05	-
6	-	0,02	-	-
12	-	0,00	0,01	0,04
18	0,11	0,02	0,07	-
24	0,09	0,23	0,29	0,38
30	0,10	0,21	0,39	0,32
36	0,15	0,10	0,29	0,49
42	0,21	0,27	0,28	0,77
48	0,30	0,28	0,80	0,95
54	0,33	0,27	2,19	1,63
60	0,15	0,24	3,37	2,01
66	0,27	0,29	1,90	2,11
72	0,32	0,42	1,93	2,31
78	0,28	0,25	1,68	2,26
84	0,48	0,39	1,93	2,70
90	0,35	0,48	1,15	1,92
96	0,40	0,22	1,82	2,94
102	0,56	0,26	2,00	1,52
108	0,31	0,44	1,41	1,38
114	0,36	0,29	1,35	1,32
120	0,43	0,29	1,80	1,14
126	0,50	0,61	1,44	1,49
132	0,39	0,36	1,30	1,54
138	0,45	0,41	1,30	1,66
144	0,55	0,52	1,55	2,02
150	0,33	0,26	1,08	1,04
162	0,53	0,37	1,84	2,00
168	0,39	0,54	1,87	1,57
174	0,39	0,37	2,02	1,53
186	0,38	0,21	1,81	1,47
192	0,39	0,33	1,56	1,37
198	0,33	0,32	1,57	1,00
210	0,41	0,30	1,07	1,31
216	0,37	0,36	1,33	1,17
222	0,29	0,27	1,19	1,00
234	0,33	0,37	1,64	0,87
240	0,36	0,40	1,14	1,19

Table B.13 - Batch fermentation in bioreactors : Variation of biomass concentration and ATP pool in CMC-xylan medium.

Fermentation time (h)	Biomass concentration (g/L)		ATP pool (mg ATP/g biomass)	
	Bioreactor 2A	Bioreactor 2B	Bioreactor 2A	Bioreactor 2B
0	3,26	2,50	0,01	-
6	2,92	2,50	-	-
12	2,71	2,53	0,00	0,01
18	2,78	2,35	0,03	-
24	2,72	2,30	0,11	0,16
30	2,83	2,57	0,14	0,19
36	3,52	3,12	0,08	0,16
42	3,42	3,07	0,08	0,25
48	3,38	3,07	0,24	0,31
54	3,02	3,25	0,73	0,50
60	3,22	3,00	1,05	0,67
66	3,21	2,78	0,60	0,76
72	2,78	3,08	0,70	0,75
78	3,28	2,81	0,51	0,80
84	3,14	2,98	0,41	0,91
90	3,44	2,84	0,33	0,68
96	3,53	3,25	0,52	0,91
102	3,55	3,19	0,56	0,48
108	3,77	3,08	0,37	0,45
114	2,96	2,68	0,46	0,50
120	3,44	2,75	0,52	0,42
126	3,72	2,74	0,39	0,54
132	3,93	3,18	0,33	0,49
138	3,69	2,75	0,35	0,60
144	3,23	2,82	0,48	0,72
150	3,38	2,63	0,32	0,39
162	3,21	2,64	0,57	0,76
168	2,90	2,48	0,65	0,63
174	3,82	2,57	0,53	0,60
186	3,19	2,45	0,57	0,60
192	2,96	2,55	0,53	0,54
198	2,98	2,65	0,53	0,36
210	2,97	2,47	0,36	0,53
216	2,99	2,44	0,45	0,48
222	2,83	2,64	0,42	0,36
234	2,66	2,33	0,62	0,37
240	2,91	2,33	0,39	0,51

Table B.14 - Batch fermentation in bioreactors : Variation of xylanase activity.

Fermentation time (h)	Newsprint medium				CMC-xylan medium			
	Bioreactor 1A		Bioreactor 1B		Bioreactor 2A		Bioreactor 2B	
	U	IU	U	IU	U	IU	U	IU
0	7,9	0,04	5,3	0,03	7,4	0,04	8,9	0,05
6	6,6	0,04	7,4	0,04	6,8	0,04	8,1	0,05
12	7,4	0,04	6,1	0,04	10,9	0,06	6,4	0,04
18	8,7	0,05	8,9	0,05	6,9	0,04	6,1	0,03
24	6,1	0,03	5,7	0,03	6,9	0,04	3,6	0,02
30	3,0	0,02	-	-	6,8	0,04	17,7	0,10
36	3,4	0,02	7,9	0,04	94,2	0,52	109,1	0,61
42	4,9	0,03	19,2	0,11	119,2	0,66	114,1	0,63
48	7,6	0,04	16,0	0,09	139,3	0,77	146,1	0,81
54	30,1	0,17	6,1	0,03	172,0	0,96	161,3	0,90
60	23,2	0,13	8,1	0,05	177,5	0,99	177,7	0,99
66	7,7	0,04	8,1	0,05	182,7	1,02	184,8	1,03
72	-	-	0,2	0,00	173,7	0,97	178,2	0,99
78	2,7	0,02	4,4	0,02	163,4	0,91	174,8	0,97
84	2,1	0,01	2,5	0,01	195,9	1,09	190,4	1,06
90	0,0	0,00	1,5	0,00	177,9	0,99	175,2	0,97
96	2,7	0,02	3,0	0,02	177,3	0,99	174,5	0,97
102	1,2	0,01	2,2	0,01	180,9	1,01	180,1	1,00
108	4,0	0,02	-	-	190,8	1,06	175,0	0,97
114	0,8	0,00	7,6	0,04	196,8	1,09	170,9	0,95
120	5,9	0,03	3,0	0,02	176,0	0,98	185,0	1,03
126	1,4	0,01	0,6	0,00	184,6	1,03	182,7	1,02
132	2,1	0,01	2,5	0,01	178,2	0,99	173,7	0,97
138	4,7	0,03	5,5	0,03	177,3	0,99	157,4	0,87
144	3,4	0,02	-	-	175,4	0,97	173,7	0,97
150	0,8	0,00	0,4	0,00	182,2	1,01	167,9	0,93
162	-	-	-	-	174,3	0,97	172,4	0,96
168	0,8	0,00	-	-	174,3	0,97	169,8	0,94
174	2,3	0,01	2,1	0,01	180,3	1,00	170,1	0,95
186	-	-	1,5	0,00	176,0	0,98	166,4	0,92
192	4,9	0,03	2,2	0,01	169,0	0,94	174,5	0,97
198	3,2	0,02	0,8	0,00	182,2	1,01	181,2	1,01
210	1,9	0,01	4,9	0,03	181,6	1,01	161,9	0,90
216	-	-	-	-	177,7	0,99	175,4	0,97
222	-	-	0,8	0,00	182,2	1,01	170,5	0,95
234	0,0	0,00	0,8	0,00	172,6	0,96	171,1	0,95
240	-	-	-	-	183,9	1,02	172,4	0,96

Definitions : 1 U = 1 µg glucose equivalent released/mL-min;
1 IU = 1 µmole glucose equivalent released/mL-min.

Table B.15 - Batch fermentation in bioreactors : Variation of CMCase activity.

Fermentation time (h)	Newsprint medium				CMC-xylan medium			
	Bioreactor 1A		Bioreactor 1B		Bioreactor 2A		Bioreactor 2B	
	U	IU	U	IU	U	IU	U	IU
0	4,7	0,03	1,4	0,01	2,7	0,02	3,2	0,02
6	2,9	0,02	1,8	0,01	3,6	0,02	1,9	0,01
12	3,0	0,01	1,8	0,01	5,4	0,03	4,2	0,02
18	2,8	0,02	2,3	0,01	1,5	0,01	-	-
24	2,6	0,01	3,1	0,02	3,4	0,02	4,2	0,02
30	3,4	0,02	3,0	0,02	6,4	0,04	6,5	0,04
36	2,2	0,01	3,6	0,02	10,0	0,06	13,4	0,07
42	3,4	0,02	5,3	0,03	14,3	0,08	13,7	0,08
48	4,1	0,02	3,2	0,02	16,0	0,09	12,7	0,07
54	6,6	0,04	3,1	0,02	19,9	0,11	12,3	0,07
60	8,1	0,05	1,1	0,01	27,3	0,15	13,9	0,08
66	4,4	0,02	8,5	0,05	18,8	0,10	18,2	0,10
72	6,6	0,04	6,4	0,04	21,6	0,12	23,8	0,13
78	6,2	0,03	7,0	0,04	25,9	0,14	24,2	0,13
84	5,2	0,03	6,7	0,04	25,3	0,14	23,1	0,13
90	5,2	0,03	5,0	0,03	23,3	0,13	20,4	0,11
96	3,5	0,02	6,2	0,03	24,9	0,14	19,2	0,11
102	4,4	0,02	4,7	0,03	21,7	0,12	24,8	0,14
108	5,9	0,03	4,9	0,03	22,9	0,13	19,2	0,11
114	4,4	0,02	5,6	0,03	23,1	0,13	22,7	0,13
120	4,5	0,03	4,8	0,03	22,3	0,12	18,0	0,10
126	5,0	0,03	4,4	0,02	21,3	0,12	20,5	0,11
132	4,8	0,03	3,9	0,02	25,3	0,14	20,8	0,12
138	2,7	0,02	3,6	0,02	19,9	0,11	21,3	0,12
144	7,7	0,04	10,3	0,06	29,9	0,17	25,9	0,14
150	6,3	0,04	9,9	0,06	27,5	0,15	23,7	0,13
162	10,3	0,06	9,8	0,06	25,7	0,14	28,7	0,16
168	7,7	0,04	10,0	0,06	27,1	0,15	24,2	0,13
174	7,0	0,04	7,8	0,04	27,6	0,15	22,7	0,13
186	11,0	0,06	16,7	0,09	31,3	0,17	18,4	0,10
192	11,2	0,06	9,3	0,05	27,1	0,15	25,8	0,14
198	8,6	0,05	7,2	0,04	20,5	0,11	19,9	0,11
210	7,2	0,04	9,0	0,05	22,1	0,12	21,9	0,12
216	6,3	0,04	6,2	0,03	26,1	0,15	17,6	0,10
222	4,6	0,03	6,2	0,03	25,6	0,14	18,4	0,10
234	6,6	0,04	8,7	0,05	18,1	0,10	15,9	0,09
240	5,0	0,03	7,2	0,04	23,9	0,13	18,2	0,10

Definitions : 1 U = 1 µg glucose equivalent released/mL-min;
1 IU = 1 µmole glucose equivalent released/mL-min.

Table B.16 - Batch fermentation in bioreactors : Variation of FPase activity.

Fermentation time (h)	Newsprint medium				CMC-xylan medium			
	Bioreactor 1A		Bioreactor 1B		Bioreactor 2A		Bioreactor 2B	
	U	IU	U	IU	U	IU	U	IU
0	2,0	0,01	1,3	0,01	1,9	0,01	1,5	0,01
6	1,9	0,01	1,8	0,01	1,9	0,01	1,9	0,01
12	2,6	0,01	2,1	0,01	1,8	0,01	0,4	0,00
18	1,3	0,01	2,8	0,02	1,6	0,01	1,9	0,01
24	1,5	0,01	2,5	0,01	1,3	0,01	1,3	0,01
30	2,4	0,01	1,2	0,01	-	-	-	-
36	0,0	0,00	2,8	0,02	-	-	-	-
42	-	-	3,6	0,02	0,2	0,00	0,3	0,00
48	0,6	0,00	0,1	0,00	0,7	0,00	-	-
54	0,0	0,00	0,3	0,00	0,2	0,00	-	-
60	1,0	0,01	-	-	-	-	2,1	0,01
66	0,2	0,00	0,0	0,00	1,5	0,01	1,0	0,01
72	0,6	0,00	1,0	0,01	2,6	0,01	2,6	0,01
78	0,5	0,00	2,0	0,01	2,0	0,01	2,5	0,01
84	0,8	0,00	1,3	0,01	3,4	0,02	3,3	0,02
90	0,9	0,01	0,8	0,00	2,1	0,01	2,7	0,02
96	0,7	0,00	0,9	0,01	1,9	0,01	1,6	0,01
102	1,3	0,01	1,1	0,01	4,3	0,02	1,1	0,01
108	2,1	0,01	1,3	0,01	2,5	0,01	3,3	0,02
114	1,0	0,01	1,5	0,01	3,8	0,02	1,5	0,01
120	0,6	0,00	1,1	0,01	2,7	0,02	2,3	0,01
126	1,9	0,01	1,8	0,01	3,1	0,02	3,6	0,02
132	0,9	0,01	1,8	0,01	1,8	0,01	2,3	0,01
138	0,5	0,00	1,2	0,01	2,0	0,01	2,3	0,01
144	1,3	0,01	-	-	-	-	0,9	0,01
150	1,2	0,01	-	-	0,6	0,00	0,3	0,00
162	-	-	0,2	0,00	-	-	0,3	0,00
168	1,3	0,01	-	-	-	-	1,1	0,01
174	0,7	0,00	0,4	0,00	0,6	0,00	-	-
186	-	-	0,9	0,01	-	-	-	-
192	0,6	0,00	-	-	0,0	0,00	-	-
198	0,6	0,00	-	-	0,6	0,00	0,6	0,00
210	-	-	0,4	0,00	1,2	0,01	-	-
216	0,8	0,00	0,2	0,00	0,5	0,00	-	-
222	-	-	-	-	0,1	0,00	0,0	0,00
234	-	-	-	-	-	-	1,0	0,01
240	-	-	0,2	0,02	-	-	-	-

Definitions : 1 U = 1 µg glucose equivalent released/mL-min;
1 IU = 1 µmole glucose equivalent released/mL-min.

Table B.17 - Batch fermentation in bioreactors : Variation of protein concentration.

Fermentation time (h)	Protein concentration (mg/L)			
	Newsprint medium		CMC-xylan medium	
	Bioreactor 1A	Bioreactor 1B	Bioreactor 2A	Bioreactor 2B
0	5	5	6	11
6	4	13	12	8
12	16	11	11	14
18	16	11	8	11
24	10	13	10	11
30	12	11	9	13
36	11	11	10	12
42	13	11	11	12
48	12	11	11	12
54	11	12	15	13
60	10	11	19	16
66	10	11	19	20
72	7	11	36	29
78	7	7	43	29
84	10	11	51	33
90	8	14	24	25
96	14	15	36	20
102	9	10	25	31
108	5	4	14	11
114	4	5	13	16
120	8	11	12	16
126	6	5	18	10
132	9	9	19	19
138	7	7	29	29
144	7	6	13	12
150	6	8	20	18
162	6	9	15	8
168	7	13	16	6
174	8	15	30	27
186	10	10	20	11
192	7	6	12	11
198	6	6	15	21
210	6	7	21	16
216	7	8	10	16
222	9	9	18	14
234	7	7	11	10
240	10	6	20	10

Table B.18 - Batch fermentation in bioreactors : Variations of $k_L a$ and $Q_{O_2} C$ in newspaper medium.

Fermentation time (h)	$k_L a$ (min^{-1})		$Q_{O_2} C$ (mg O_2/L-min)	
	Bioreactor 1A	Bioreactor 1B	Bioreactor 1A	Bioreactor 1B
0	1,628	1,928	0,020	
24		0,191		
36	0,274		0,008	
48		0,248		0,057
67	0,254		0,048	
72		0,219		0,072
84	0,182		0,019	
96	0,368	0,470	0,034	0,095
108			0,034	
120		0,177		0,061
132			0,029	
144		0,376		0,035
167			0,067	0,037
188		0,153		0,037
191	0,178		0,039	
214	0,087	0,144	0,049	0,033
238	0,428	0,360	0,039	0,049

Table B.19 - Batch fermentation in bioreactors : Variations of $k_L a$ and $Q_{O_2} C$ in CMC-xylan medium.

Fermentation time (h)	$k_L a$ (min^{-1})		$Q_{O_2} C$ (mg O_2/L-min)	
	Bioreactor 2A	Bioreactor 2B	Bioreactor 2A	Bioreactor 2B
0	1,701	0,877	0,024	0,003
12	1,948		0,015	
24		0,129		0,065
36	0,194		0,079	
48			0,000	
60			0,095	0,110
72			0,094	
84		0,091		0,064
96	0,155		0,057	
108		0,127		0,061
132	0,127		0,053	
144		0,123		0,057
164	0,112	0,154	0,040	0,049
186	0,087	0,159	0,030	0,022
210	0,062	0,157	0,047	0,044
236	0,095	0,105	0,057	0,024

APPENDIX C

IDENTIFICATION OF ACIDOPHILIC FUNGUS

ATCC REPORT

SINCE 1925 AN INDEPENDENT NON PROFIT ORGANIZATION DEVOTED TO THE PRESERVATION AND DISTRIBUTION OF REFERENCE CULTURES

American
Type Culture
Collection

12301 PARKLAWN DRIVE · ROCKVILLE, MARYLAND 20852-1776 USA · (301) 881-2600 TELEX: ATCCROVE 908-768

June 6, 1985

Dr. Anh Duy
Associate Professor
Lavel University
Department of Chemical Engineering
Sainte Foy
Quebec G1K 7P4
Canada

14 JUIN 1985

Dear Dr. Duy:

Enclosed please find a Project Report on the identification of your culture.

A billing invoice will be sent to your attention under separate cover.

If you have any questions or if you have further need of our services, please feel free to call.

Sincerely,

Judith Franke

Judith Franke
Applied Sciences
Microbiologist

JF:rga

Enclosure

AFFILIATED ORGANIZATIONS
AMERICAN ASSOCIATION OF IMMUNOLOGISTS AMERICAN INSTITUTE OF BIOLOGICAL SCIENCES AMERICAN PHYTOPATHOLOGICAL SOCIETY AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS
AMERICAN SOCIETY FOR CELL BIOLOGY AMERICAN SOCIETY FOR MICROBIOLOGY AMERICAN SOCIETY OF PARASITOLOGISTS AMERICAN SOCIETY OF ZOOLOGISTS
AMERICAN SOCIETY OF TROPICAL MEDICINE AND HYGIENE CANADIAN FEDERATION OF BIOLOGICAL SOCIETIES GENETICS SOCIETY OF AMERICA INFECTIOUS DISEASES SOCIETY OF AMERICA

American Type Culture Collection
12301 Parklawn Drive • Rockville, Maryland 20852

PROJECT REPORT

Title: Identification of Acidophilic Fungus
Patent Strain ATCC 20677

Date: June 6, 1985

Reference: SC 922

Submitted to: Dr. Anh Duy
Associate Professor
Lavel University
Department of Chemical Engineering
Sainte Foy
Quebec G1K 7P4
Canada

PROJECT REPORT

Culture Identification

SC 922

Isolate submitted as: ATCC 20677 Patent Strain

Identified as: *Penicillium ochro-chloron* Biourge

Morphology:

Conidial chains strongly divergent and becoming tangled in age, not forming columns.

Conidia usually roughened.

Vegetative hyphae uncolored or in slightly buff shades.

Colony reverse is colorless or in a slightly yellow shade.

Media used for evaluation of ATCC 20677 and the comparison with ATCC 10540.

1. Czapek's agar

$NaNO_3$	3.0 g
K_2HPO_4	1.0 g
$MgSO_4$-$7H_2O$	0.5 g
KCl	0.5 g
$FeSO_4$-$7H_2O$	0.01g
Sucrose (commercial grade)*	30.0 g
Agar	15.0 g
Distilled water	1.0 L

*Add sucrose prior to dispensing.

2. Czapek's agar with yeast extract

3. Harrold's M40Y

Malt Extract	20.0 g
Yeast Extract	5.0 g
Sucrose	400.0 g
Agar	20.0 g
Distilled water	1.0 L

4. Malt agar (DIFCO 0024)

5. Malt extract agar (Blakeslee's Formula)

Malt Extract	20.0 g
Glucose	20.0 g
Peptone	1.0 g
Agar	20.0 g
Distilled water	1.0 L

6. Potato dextrose agar (PDA)

Diced Potatoes	300.0 g
Glucose	20.0 g
Agar	15.0 g
Distilled water	1.0 L

Boil finely diced potatoes in 500 ml of water until thoroughly cooked; filter through cheesecloth and add water to filtrate to 1.0 L. The agar is dissolved in the filtrate by heating, and the glucose is added prior to sterilization.

References:

Manual of the Penicillia. Raper and Thom. 1949. Williams and Wilkens.

Manual and Atlas of the Penicillia. Carlos Ramirez. 1982. Elsevier Biomedical.

The Genus Penicillium and its telemorphic staes Eupenicillium and Talaromyces. John I. Pitt. 1979. Academic Press.